MATERNAL PHYLOGEOGRAPHY OF BROWN BEARS (*URSUS ARCTOS*) AND TESTING THE UTILITY OF NON-INVASIVE GENETIC SAMPLES

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MATERNAL PHYLOGEOGRAPHY OF BROWN BEARS (*URSUS ARCTOS*) AND TESTING THE UTILITY OF NON-INVASIVE GENETIC SAMPLES

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ABSTRACT

MATERNAL PHYLOGEOGRAPHY OF BROWN BEARS (*URSUS ARCTOS*) AND TESTING THE UTILITY OF NON-INVASIVE GENETIC SAMPLES

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The genetic diversity and phylogeography of brown bear maternal lineages have been studied extensively over the last two decades. In this study the genetic diversity and maternal phylogeography of non-invasively sampled 35 brown bears, including 5 captive individuals were reported from Turkey. In addition to the optimization of DNA extraction from hair, faeces and old skin samples and their PCRs, Bayesian phylogenetic analyses based on a 269 bp long piece of bear mitochondrial DNA were conducted and 14 novel haplotypes belonging to three major lineages were revealed. The most widespread lineage was found to be the "Eastern" clade 3a, while geographically more restricted "Western" and "Middle Eastern" lineages were reported for the country for the first time. A specimen from the Taurus range (southern Turkey) was shown to be closely related to the presumably extinct bears in Lebanon. Moreover, a unique novel lineage that appears to have split early within the Middle Eastern clade was defined. Despite limited sampling, this study demonstrates a high level of mitochondrial diversity in Turkish brown bears, extends the ranges of both

European and Middle Eastern clades into Turkey, and identifies a new divergent lineage of possibly wider historical occurrence while demonstrating the significance of non-invasive genetic sampling for such analysis.

Keywords: brown bear, mtDNA control region, non-invasive genetic sampling, phylogeography

ÖZ

AYILARIN (*URSUS ARCTOS*) ANA SOY FİLOCOĞRAFYASI VE GİRİŞİMSİZ GENETİK ÖRNEKLERİN KULLANILABİLİRLİĞİNİN TEST EDİLMESİ

Çilingir, Fatma Gözde

Yüsek Lisans, Biyoloji Bölümü

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Son 20 yılda, boz ayı ana soylarının genetik çeşitliliği ve filocoğrafyası üzerine çeşitli çalışmalar yapılmaktadır. Bu çalışmada Türkiye'deki boz ayı ana soylarının genetik çeşitliliği ve filocoğrafyası girişimsiz olarak örneklenmiş 5'i hayvanat bahçelerinden olmak üzere 35 tane boz ayı örneği ile rapor edilmiştir. Kıl, dışkı ve post örneklerinden DNA izolasyonu ve bu örneklerin PCR'lerinin optimizasyonunun yanı sıra, 269 bp'lik mitokondriyel DNA kontrol bölgesine dayanan Bayezyen filogenetik analizi uygulanmış ve sonuç olarak üç farklı ana soya ait 14 farklı haplotip bulunmuştur. En yaygın ana soy olarak, "Doğu Ana Soyu", ana soy 3a bulunurken, coğrafi olarak daha sıkışık bir biçimde olduğu tespit edilen "Batı Ana Soyu" ve "Orta Doğu Ana Soyu" Türkiye'de ilk defa tespit edilmiştir. Toroslar'dan alınmış bir örneğin ise önceden neslinin tükendiği söylenen Lübnan ayıları ile yakın akraba çıktığı gösterilmiştir. Tüm bunlara ek olarak "Orta Doğu Ana Soyu'ndan erken dönemde ayrıldığı görülen yeni bir ana soy da tespit edilip tanımlanmıştır. Kısıtlı örnek miktarına rağmen, bu çalışmada Türkiye'de bulunan boz ayı ana soylarının genetik çeşitliliğinin hayli

yüksek olduğu gösterilmiş; Avrupa ve Orta Doğu ana soylarının öngörülen dağılım aralıkları genişletilmiş; genetik çeşitliliği yüksek olan ve büyük ihtimalle geçmişte daha geniş alanlara yayılmış bir şekilde bulunan bir yeni ana soy tespit edilmiştir. Dolayısıyla tüm bu tespitlerin yapılabilmesi için girişimsiz genetik örneklemenin önemi bir kez daha vurgulanmıştır.

Anahtar kelimeler: boz ayı, mtDNA kontrol bölgesi, girişimsiz genetik örnekleme, filocoğrafya

To the dreams we share with my beloved C. Ozan...

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I would like to thank my greatly beloved grandparents Fikriye KİŞMİR, Hakkı KİŞMİR; my parents Hülya ÇİLİNGİR, Fikri ÇİLİNGİR; my brothers A. Altuğ ÇİLİNGİR, H. Serbülent ÇİLİNGİR and my brothers' dear wives, Aylin ÇİLİNGİR and Merve ÇİLİNGİR for their infinite love, care and confidence that I have felt deeply behind throughout my life. Also, I have special thanks and lots of kisses to my little beautiful nieces Arya ÇİLİNGİR and A. Ilgaz ÇİLİNGİR for their existence and for the beauty in their eyes promising infinite love and loveliness.

Finally, I unconditionally express gratitude to my immortal beloved Cemil Ozan CEYHAN not only for the sensitive connection we have, but also for the cooperation in challenging periods of life without hesitation. Nothing would and will definitely be possible without his warm embrace…

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LIST OF ABBREVIATIONS

Akaike Information Criterion: AIC

Ancient DNA: aDNA

Base pairs: bp

Bayesian Information Criterion: BIC

Control region: CR

cytochrome b: *cytb*

Decision Theory Method: DT

Degree Celsius: ^{o}C

Deoxyribonucleic acid: DNA

Deoxyribonucleotide triphosphate: dNTP

Dichlorodiphenyltrichloroethane: DDT

Distilled Water: dH_2O

Dynamical Likelihood Ratio Test: dLRT

Ethylene diamine tetra acetic acid: EDTA

Guanidinium thiocyanate: GuSCN

Haplotype Diversity: h

Hierarchical Likelihood Ratio Test: hLRT

Identity: ID

Molar: M

Magnesium Chloride: $MgCl_2$

Microliter: µl

Milligram: mg

Milliliter: ml

Millimeter: mm

Millimolar: mM

Minute: min

Mitochondrial DNA: mtDNA

Nanometer: nm

Nuclear DNA: nDNA

Nucleotide diversity, pi: π

Optical Density: OD

Picomoles: pmol

Polymerase Chain Reaction: PCR

Revolutions per minute: rpm

Second: s

Time to the Most Recent Ancestor: TMRCA

Tris- Acetate- EDTA: TAE

Tris- EDTA: TE

Ultraviolet: UV

CHAPTER 1

INTRODUCTION

1.1 General Biology of Brown Bear

1.1.1 Systematics of brown bear

Brown bear (*Ursus arctos L.*) belongs to Phylum Chordata, Class Mammalia, Order Carnivora, Family Ursidae, Subfamily Ursinae, Genus *Ursus* (McLellan *et al.*, 2008). The genus *Ursus* comprises four distinct species: *U. thibetanus* (Asiatic black bear), *U. maritimus* (polar bear), *U. americanus* (American black bear) and *U. arctos* (brown bear, grizzly bear, Mexican grizzly bear).

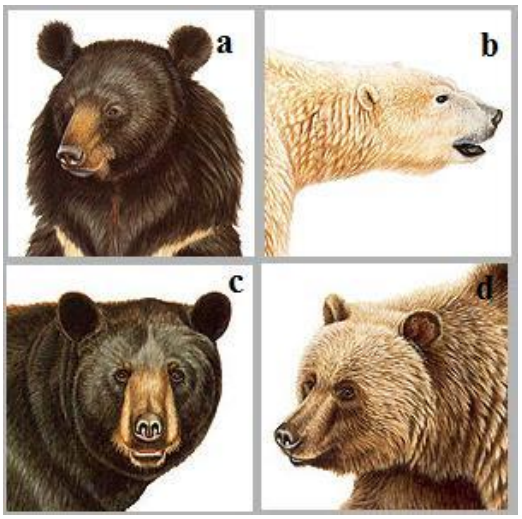

Figure 1.1: Four species belonging to Genus *Ursus*: a) *U. thibetanus* b) *U. maritimus* c) *U. americanus* d) *U. arctos* (IBA, 2007)

1.1.2 Morphological features of the species

Brown bears are large-bodied members of Order Carnivora, Family Ursidae. They are the biggest extant mammal species in Turkey (Ambarli & Bilgin, 2008).

Brown bears have a massive body with a colossal head, relatively indistinct eyes because of their small size, small rounded ears, a short nose and a short tail (Pasitschniak-arts, 1993). Although the name implies 'brown' colour, the coat varies between the spectrum of black and white including all varieties of brown (Jonkel, 1994; Pasitschniak-arts, 1993). Ambarlı (2006) stated that brown bears found in Artvin (north-eastern Turkey) might have a dark stripe on the back of the animal starting from the posterior side of the head and running through the middle of the back.

When the general morphology of brown bears are compared with that of polar bears, the most distinctive feature that differs is the colour of the fur. Moreover, the neck of the polar bear is distinctively longer than any other bear species (IBA, 2007). On the other hand, the cave bear (*Ursus spelaeus*), an extinct form closely related to brown bears, is comparable with the largest modern day bear while having humerus (upper bone in the forearms) similar to polar bears and femur (longest bone in the hind leg) similar to Kodiak bears (Christiansen, 1999).

Although the size of brown bears throughout the world may differ, male brown bears are almost twice heavier than female bears (Jonkel, 1994). In Turkey, the mean weights calculated from hunted bears in Artvin in 1995 are 191.43 kg for mature males (n=7) and 136.25 kg for mature females (n=4) (Ambarlı, 2006a).

1.1.3 Ecology, habitat and ontogeny of the species

Although brown bears are recognised as omnivorous, in Turkey they seem to prefer mostly a herbivorous diet (Ambarli, 2006). Can (2004) reported that the main diet of bears in Turkey consists of acorns (*Quercus*), beechnuts (*Fagus*), chestnuts (*Castanea*), hazelnuts (*Coryls*), plums (*Prunus*), wild apples (*Malus*), wild pear (*Pyrus*), bilberry, ants, bees, wasps and occasionally livestock.

Because bears are opportunists, anthropogenic food sources obtained from e.g. garbage dumps, trash cans and bee yards may be present in bear diet (Jonkel, 1994).

By definition habitat of brown bears should provide enough energy for reproduction, breeding and survival as well as include secure places for denning (Swenson *et al.,* 2000; Jonkel, 1994). Human alterations within the last several centuries forced a contraction in range and occurrences in less suitable habitats (Swenson *et al.,* 2000). If human alterations would be eliminated, brown bears' potential habitats would cover steppes, northern and alpine tundra in addition to deciduous and coniferous forests (Jonkel 1994; Swenson *et al.,* 2000). Can & Togan (2004) stated that in Turkey, brown bear habitat is restricted to forest and untouched habitats in Ankara, Antalya, Artvin, Bingöl, Bitlis, Bolu, Bursa, Çanakkale, Çankırı, Elazığ, Erzurum, Giresun, Gümüşhane, Hakkari, Isparta, Kars, Kastamonu, Malatya, Muğla, Muş, Ordu, Sakarya, Siirt, Sivas, Şırnak, Tokat, Trabzon, Tunceli, Van, and Zonguldak provinces including a small population in Muğla region, between Köyceğiz and Marmaris. Habitat overlap of brown bear with other mammal species such as wild boar, red fox, gray wolf, wild goat and chamois was reported by Ambarlı (2006a).

Home range size of brown bears is directly related with food availability, but sex, age, condition of the animal, distance to denning sites, proximity to breeding individuals etc. also influence the size of the home range (Pasitschniak-arts, 1993). Males have larger home range sizes than females (Jonkel, 1994).

Brown bears are polygamous, meaning that males can breed with several females and vice versa (Pasitschniak-arts, 1993). Breeding occurs from May to July, and births occurring from January to March following a 180 to 266 days long gestation. Births usually occur while the female is still in hibernation. Females normally bore two or three offspring, and breeding may occur again 2 to 4 years later (WWF, 2009).

Hibernation is one of the intriguing features of the brown bears. It is a period when brown bears do not consume any food or drink, do not urinate or defecate but they maintain almost the same body temperature throughout (Jonkel,

1994). Bears hibernate when food is not readily available; dormancy state takes 3-7 months and starts between October- December and lasts till March- May of the next year (Pasitschniak-arts, 1993).

1.1.4 Behaviour of the species

Because female brown bears look after their cubs, they can be observed with them while male brown bears are usually alone; in general brown bears may be considered solitary species (IBA, 2007). In the breeding season, the solitary structure of their social system holds but a male's home range covers home ranges of several females in order to increase its chances of breeding. In addition, aggregations of brown bears may also occur around readily available rich food sources (IBA, 2007; WWF, 2009). On the other hand, it is worthy to note that brown bears do not show territorial defence (Servheen, 1990).

Brown bears communicate largely on olfaction as well as sight and sound. Excoriating, biting, scratching, and stripping tree bark can be annotated as visual and olfactory traces for marking territory, showing sexual phase, or can serve as grooming stakes (Pasitschniak-arts, 1993).

1.1.5 Distribution of the species in the world

The most broadly distributed bear in the world is *Ursus arctos*. The species ranges in North America, Europe, Asia, The Middle East and even North Africa (Swenson *et al.,* 2000).

In Russia it is believed that they number more than 100,000. For U.S. estimates predict approximately 33,000 individuals whereas 25,000 bears are estimated for Canada and 14,000 for Europe (McLellan *et al.,* 2008).

Sound estimates on the number of bears in Turkey do not exist yet. According to Servheen (1999), Artvin, Hakkari, Tunceli and Erzincan host the highest populations of brown bear in Turkey. Ambarlı (2006a) estimated a population density of 25 adults per 100km^2 in the Özgüven Valley, Artvin. Crude estimates

on the total number of brown bears in Turkey are 3000 individuals (Can 2004) and 4000 indidivuals (C. Bilgin, pers.comm).

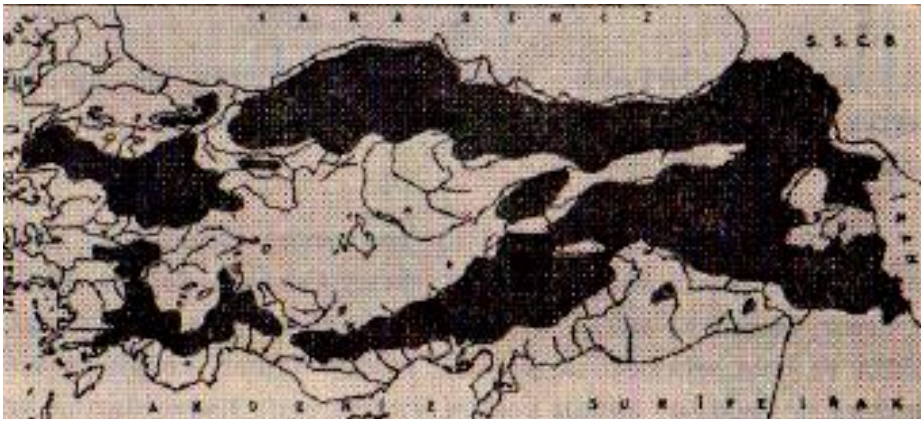

Figure 1.2: Approximate distribution of brown bears in Turkey (Turan, 1984)

1.1.6 Brown bear studies in Turkey

Brown bear studies in Turkey can be summarised within the frame of ecology and the general status of the animals. For example an M.Sc. thesis titled "The Status of gray wolf (*Canis lupus* L. 1758), brown bear (*Ursus arctos* L. 1758) and Eurasian lynx (*Lynx lynx* L. 1758) in Turkey and recommendations for effective conservation program", submitted by Can (2001) summarised main threats, limiting factors and conservation measures proposed on brown bears. Also some suggestions for brown bear research were proposed by Can & Togan (2004).

In a camera trapping study in Yenice Forests conducted by Can (2006), brown bears were also captured but no calculations of population density estimates were made for the brown bears as were done for other study species.

In an M.Sc. thesis submitted by Ambarlı (2006a), levels of conflict between human and brown bears in Yusufeli-Artvin/Turkey were investigated. This study reported the levels of human-bear conflict, evaluated local human attitudes and responses to the conflict, and provided preliminary data on density, habitat use and daily activity patterns of the study species. Based on this study, Ambarlı (2006b) reported the live capture, radio-collaring and

release of the first Turkish bear ever, as well as reported a first population density estimate.

In 2008, population viability modelling with harvesting scenarios for a brown bear population at Yusufeli,Artvin, Turkey was conducted by Ağzıtemiz (2008). In this study, it was revealed that while poaching pressure on the study population continues, even low levels of trophy hunting may produce risks for the population. Also, in the same year, Ambarlı and Bilgin (2008) published a study that summarised the conflict between human and brown bears and gave recommendations for reducing conflict levels, through techniques such as using electric fences.

Besides ecological investigations on brown bears, there is also a study conducted by Ambarlı (2010a) that compared the perception of brown bear by students in either rural or urban areas. The study found that the perception on poaching and conservation differed among students dependeing on background. It also found that despite being fearful of bears, students had positive ideas about this species.

A short communiation conducted by Ambarlı (2010b) reported on the marking behaviour of brown bear. The marking behaviour which includes biting, chewing, clawing and rubbing was found to vary among brown bears and the reasons of this situation were discussed.

Finally, through the capture and radio-tracking of several more bears (2010c, 2012) in Yusufeli, Artvin, new information on home range and habitat use of bears were obtained. This study found out that bears in the study region had an unusually high population density, females and males had relatively small home ranges, averaging 20 and 131 sq. km., respectively. The high density was possible due to bears making use of highly productive sources such as oak mast, abandoned pastures and orchards, and due to the high tolerance showed by the local people.

1.2 Phylogeography of Brown Bear in the Palearctic

For brown bears, there are several genetically and/or geographically distinct clades and subclades in the Palearctic. In this section, the nomenclature proposed by Leonard *et al.,* 2000 and improved by Miller *et al.,* 2006 and Calvignac *et al.*, 2008; 2009, which basically includes clade names numbered 1-6 or including geographical provenances (i.e North Africa, Iran) will be used. Moreover, brown bear clades found in the Palearctic will be examined in terms of their recent distribution in different regions.

Figure 1.3: Approximate distribution of brown bear clades and subclades. The number '49' refers to the individual belonging for the Iran clade (Miller *et al.,* 2006).

The identification of different clades of brown bears is generally based on mitochondrial DNA (mtDNA) control region (CR) sequences as well as some supporting information on cytochrome b (*cyt b*) sequences (Davison *et al.*, 2011). It can be claimed that genetic diversity of the brown bear was well studied throughout the Holarctic. In addition to mitochondrial variation surveys on North American brown bears (Talbot & Shields, 1996; Waits *et al.,* 1998; Miller *et al.*,, 2006) two different matrilineal lineages of brown bears were shown to exist in Europe. Clade 1 was found in western Europe with subclade 1a in Cantabrian Mountains- Spain, Pyrenees- France and southern Scandinavia, and subclade 1b in southern and eastern Europe (Bosnia, Bulgaria, Croatia, Greece, Italy, Poland, Romania, Slovenia) (Taberlet & Bouvet, 1994). Also, a second matrilineal lineage, clade 3a was found in northern and eastern Europe (Estonia, Finland, northern Scandinavia, Slovakia, Romania

and Russia (Kohn *et al.*,1995; Korsten *et al.*, 2009; Taberlet & Bouvet, 1994). There are contact zones in Europe in which subclades and clades were observed together (in Carpathians, Kohn *et al.*,1995; Zachos *et al.*, 2008; in Scandinavia, Taberlet *et al.*, 1995). Following further research, the distribution of Eastern European lineage, clade 3a was enlarged from the Caucasus east to Siberia (Korsten *et al.*, 2009; Murtskhvaladze *et al.,* 2010).

In Inner Asia, there are two exceptional lineages in Mongolia (Gobi Desert) and Pakistan, named Clade 6, and in the Tibetan Plateau, named Clade 5 (Masuda *et al.*, 1998; Matsuhashi *et al.*,1999, 2001 and Miller *et al.*, 2006). Studied mtDNA markers showed that the lineage found in Mongolia is closer to the Western European lineage (clade 1), whereas the Tibetan lineage was found to be closer to the Eastern Europe lineage (clade 3a) (Masuda *et al.*,, 1998; Matsuhashi *et al.*,, 1999, 2001). On the other hand, on the Hokkaido Island of Japan, two geographically and genetically distinct lineages were identified; one of them belongs to clade 3a and the other two belong to clade 3b and clade 4 whose sister clades are now found in North America (Masuda *et al.*,, 1998; Matsuhashi *et al.*, 1999 and Korsten *et al.,* 2009).

In the Middle East, first Miller *et al.,* (2006) identified a different individual from Iran (individual 49). Later a divergent Iranian clade was identified by Calvignac *et al.,* (2009) with additional ancient Lebanon samples which are genetically closer to Western European lineage. Moreover, Calvignac *et al.,* 2008 found an extinct and highly divergent clade restricted to North Africa. For Turkish bears, so far mtDNA information (cytb sequences) from only two individuals from Artvin are available (Talbot & Shields, 1996) and both individuals belong to the same haplotype within clade 3a (see Fig.1.3 for geographical distribution of brown bears in Holarctic).

In order to comment on phylogeographic processes, it is necessary to determine time-scales for genetic diversity and integrate genetic diversity patterns with evolutionary processes (Davison *et al.,* 2011). There are several methods for molecular clock calibration of the splits between brown bear clades. For example, researchers initially assigned nucleotide substitution rates taken from another species (i.e humans, Taberlet & Bouvet, 1994) because of a lack of fossil samples of brown bears. However, this method is inappropriate because

every species has its own substitution rate (Davison *et al.*, 2011). When paleontological data became available for brown bears, substitution rates derived from interspecific comparisons (external calibration) (i.e brown-cave bears (Hofreiter *et al.*, 2002) were used for the estimation of divergence times of different brown bear clades. However, one of the drawbacks of this method was proposed to be an overestimation of divergence times due to the existence of transient polymorphisms that do not become fixed in populations (Ho *et al.*,, 2007; 2008). Alternatively, one can use an internal calibration method, in which time of most recent ancestors (TMRCA) of clades is calculated based on sequence divergence among modern sequences and reliably-dated ancient ones. Although this method gives far more accurate dates than external calibration, challenges with ancient DNA and the 'age' limit of fossil calibration restrict its use (Davison *et al.*, 2011).

When the drawbacks of internal and external molecular calibrations are considered, one cannot decide which method to use. However, there are some studies such as Korsten *et al.,* 2009's, which aimed to integrate external calibration and internal calibration with a technique called multiple calibration.

As a demonstration of how TMRCA estimations may vary between the three methods above, a comparison of the estimates can be seen in the table below.

Table 1.1: Different molecular clock estimations of time to the most recent ancestors of brown bear clades (modified from Davison *et al.,* 2011). a) Multiple calibration estimates from Davison *et al.,* 2011 b) Multiple calibration estimated from Korsten *et al.,* 2009 c) Internal and external calibration estimates from (Ho *et al.*,, 2008)

Node Description	**Node Age (kyBP)**			
	Multiple Calibration[a]	**Multiple Calibration[b]**	**Internal Calibration[c]**	**External Calibration[c]**
All sampled brown bears in the studies	263 (162-400)	193 (114-325)	211 (143-295)	1,159 (745-1,622)
3a / 3b	92 (51-133)	-	35 (11-75)	374 (89-741)
3 / 4	140 (87-213)	-	75 (40-130)	458 (167-755)
Clade 1 / Extant Europe, Middle East	100 (49-164)	81 (45-144)	74 (45-109)	415 (209-659)
1a / 1b	>63 (40-98)	-	-	-

1.3 Mitochondrial DNA Control Region Marker

The mitochondrial genome can be divided into three sections: a large coding region responsible for the production of the biological molecules required in the energy producing processes and including tightly joined genes with few or no spacer nucleotide in between (Gray, 1989), and a small highly polymorphic control region hosting hypervariable sites (Gray, 1989; Hoong & Lek, 2005) (see Fig.1.4 for a sample Ursidae complete mtDNA sections and universal primers). When the mutation rate of mitochondrial DNA (mtDNA) is compared with nuclear DNA (nDNA), it can be claimed that mtDNA mutation rate can be ten times higher than the rate for nDNA (Hoong & Lek, 2005).

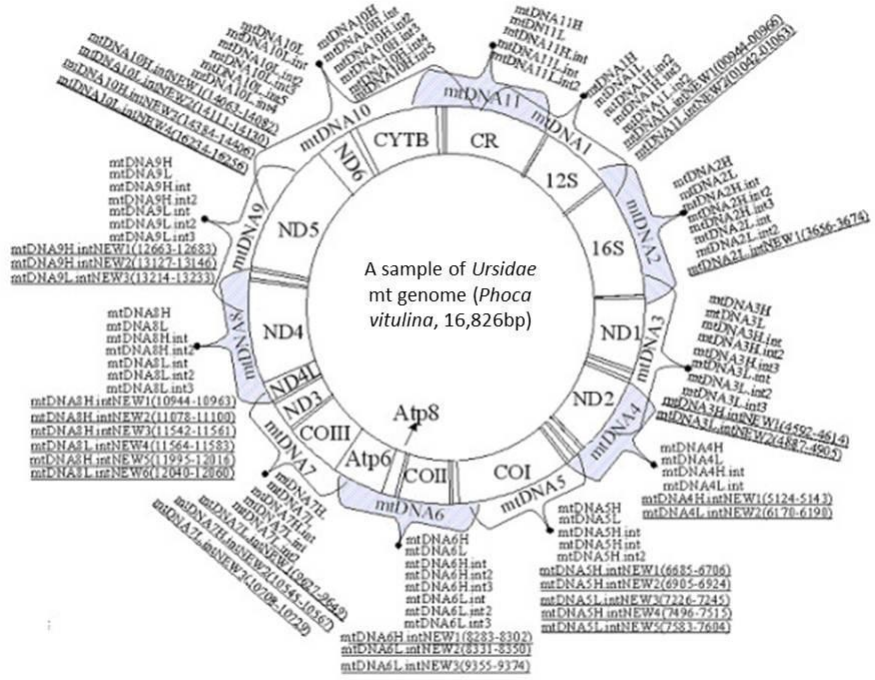

Figure 1.4: Complete Ursidae mtDNA genome illustration, showing the sections of the mtDNA (Delisle & Strobeck, 2002) and the universal primers (the ones without underlines) (Figure was taken from (Yu *et al.*, 2007).

For animal phylogenetic/phylogeographic studies, there are several different marker systems available. For example, for the characterization of female lineages mtDNA markers; of male lineages in mammals, Y-chromosome specific markers or of both female and male combined lineages, microsatellites can be

preferred. However, there are several advantages of the preference of mtDNA markers, such as: the ease of the amplification of mtDNA because of high copy numbers in the cell; the conserved structure of mtDNA (few duplications, no introns, no recombination); the high mutation rate of mtDNA which bears the high variability among the natural populations; the conserved structure of highly variable sites (i.e control region) which eases the primer designing process (Harrison, 1989; Yu *et al.*, 2007; Galtier *et al.*, 2009).

In the brown bear case, conducting wide-scale phylogeographic studies via mtDNA marker systems has an additional advantage, because the characterization of maternal lineages of a species with female philopatry may increase the possibility of capturing ancient phylogeographic processes, mainly due to the slower change in female-specific markers than autosomal biallellic genetic markers (Zedrosser *et al.*, 2007; Davison *et al.,* 2011).

The most frequently used mtDNA marker is the control region (CR) so far (Taberlet & Bouvet, 1994; Calvignac *et al.*, 2009; Kohn *et al.*, 1995; Korsten *et al.*, 2009; Murtskhvaladze *et al.*, 2010; Saarma *et al.*, 2007 etc.). Although the rapid mutation rate in the hypervariable sites of the control region makes this marker a useful one, possible homoplasmy may lead to error prone results (Korsten *et al.,* 2009). Besides the control region, there are also some studies which have used the –relatively slowly changing- cytochrome b (*cytb*) sequences either for phylogenetic analysis or for supporting CR based analysis (Talbot & Shields, 1996, Calvignac *et al.,* 2008). In the figure below, the positions and the structure of cytb and CR can be seen.

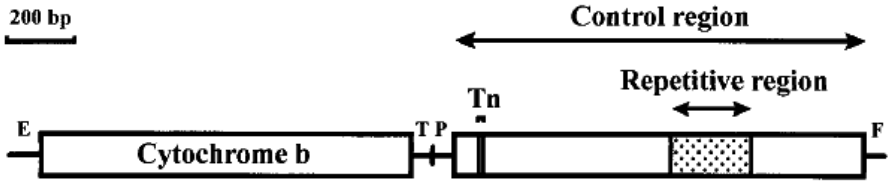

Figure 1.5: Position of cytochrome b and control region with the structure of CR (taken from Matsuhashi *et al.*,1999).

In this study, a 269 bp long segment of the 1.2 kb control region of the brown bear mtDNA was used. Taberlet & Bouvet (1994) suggested the region flanking the Pro-tRNA gene because enough variations are represented by this region to characterize relationships among different brown bear populations.

1.4 Aim of the Study

There are two aspects that were encompassed by this study. First one is methodological aspect aiming to optimize non-invasively and invasively collected samples' mtDNA extraction and amplification procedures. The second aspect (the phylogenetic/phylogeographic aspect) aims to identify recent distinct brown bear matrilineal lineages (clades) found in Turkey, to evaluate their genetic diversity, and to comment on patterns of brown bear phylogeography in Turkey.

CHAPTER 2

MATERIALS AND METHODS

2.1 Sample Collection

Non-invasive genetics is an advantageous approach allowing genetic studies to be carried out without catching, disturbing or even observing the target individuals (Waits & Paetkau, 2005). Since 1990s, non-invasive genetic sampling studies have been conducted by extracting genetic material from hair, faeces, or other DNA sources from numerous groups of animals found in different regions in the world (see Table 1 in Beja-Pereira *et al.*, 2009). Non-invasive genetic sampling was also used in some studies in Turkey in which wild goats and Anatolian mouflon were the target species (Balkız, 2002; Kayım, 2005).

In this study, 47 hair samples, 49 scat samples and 15 tissue samples (9 old skin and 6 fresh tissue samples) were obtained from different parts of Turkey. Most of the samples were collected from the northeast of Turkey where the species is most numerous (Table 2.1). Hair samples were collected via hair traps consisting of barbed wire surrounding a scent lure, from fences around agricultural fields and bee yards, from rubbing trees, or from live captures (Woods *et al.*, 1999). Scat samples were collected opportunistically between 2005 and 2011. In addition, fresh scat samples were obtained from several bears in captivity, although the exact origins (within Turkey) of only some of those individuals were known. Old skin samples from specimens that were hunted during the years when bear hunting was legal in Turkey were provided by private collectors. Small pieces of fresh tissue were obtained from live captures that were under anesthesia during fieldwork for a separate telemetry study conducted by Ambarlı *et al.* (2010c).

Hair samples were preserved in dry envelopes until DNA extraction as suggested in Gagneux *et al.* (1997) and Woods *et al.* (1999). Sample IDs were created in regard to the DNA source and the number of samples. For instance, for hair samples the first two letters of the IDs were annotated as BF and enumeration was done in parallel with the number of samples (BH1-BH47; Table 2.1).

Table 2.1: Sample IDs belonging to hair samples were shown with their date of collection and location. Locations of the samples were given with province, city where applicable.

Sample ID	Date of Collection	Location
BH1	July 2011	Yusufeli, Artvin
BH2	July 2011	Yusufeli, Artvin
BH3	2010	Yusufeli, Artvin
BH4	September 2011	Yusufeli, Artvin
BH5	September 2011	Yusufeli, Artvin
BH6	September 2011	Yusufeli, Artvin
BH7	September 2011	Yusufeli, Artvin
BH8	September 2011	Yusufeli, Artvin
BH9	March 2011	Yusufeli, Artvin
BH10	May 2010	Merkez, Artvin
BH11	August 2006	N/A, Artvin
BH12	October 2011	Yusufeli, Artvin
BH13	October 2012	Yusufeli, Artvin
BH14	October 2013	Yusufeli, Artvin
BH15	September 2005	Yusufeli, Artvin
BH16	July 2008	Yusufeli, Artvin
BH17	July 2008	Yusufeli, Artvin

Table 2.1 (cont'd)

Sample ID	Date of Collection	Location
BH18	August 2008	Yusufeli, Artvin
BH19	August 2008	Yusufeli, Artvin
BH20	June 2008	Yusufeli, Artvin
BH21	July 2009	Yusufeli, Artvin
BH22	May 2010	Yusufeli, Artvin
BH23	May 2010	Yusufeli, Artvin
BH24	May 2010	Merkez, Artvin
BH25	August 2008	Borçka, Artvin
BH26	2010	Yusufeli, Artvin
BH27	September 2010	Yusufeli, Artvin
BH28	September 2010	Yusufeli, Artvin
BH29	September 2010	Yusufeli, Artvin
BH30	September 2010	Yusufeli, Artvin
BH31	2009	Yusufeli, Artvin
BH32	June 2010	Yusufeli, Artvin
BH33	September 2010	Yusufeli, Artvin
BH34	2010	Yusufeli, Artvin
BH35	September 2010	Yusufeli, Artvin
BH36	May 2010	Yusufeli, Artvin
BH37	June 2011	Yusufeli, Artvin
BH38	September 2010	Not stated
BH39	July 2011	Yusufeli, Artvin
BH40	August 2010	Çaykara, Trabzon
BH41	July 2010	Yusufeli, Artvin
BH42	July 2011	Şavşat, Artvin

Table 2.1 (cont'd)

Sample ID	Date of Collection	Location
BH43	July 2011	Şavşat, Artvin
BH44	July 2011	Yusufeli, Artvin
BH45	June 2011	Çamlıhemşin, Rize
BH46	June 2011	Yusufeli, Artvin
BH47	July 2011	Yusufeli, Artvin

Faeces samples were all preserved in 95% ethanol (Beja-Pereira *et al.,* 2009; Murphy *et al.*, 2002), for faeces samples BF1-BF49 were used (see table 2.2).

Table 2.2: Sample IDs of faeces samples used in this study was given with their date of collection, with the distinction of wild of captured bear sample and with the location information including province and city where applicable. Some of the captive bear samples' locations were not known; therefore the approximate locations were stated.

Sample ID	Date of Collection	Wild	Location
BF1	July 2011	yes	Şavşat, Artvin
BF2	July 2011	Yes	Şavşat, Artvin
BF3	July 2011	Yes	Şavşat, Artvin
BF4	July 2011	Yes	Şavşat, Artvin
BF5	July 2011	Yes	Şavşat, Artvin
BF6	June 2011	Yes	Çamlıhemşin, Rize
BF7	May 2011	Yes	Nallıhan, Ankara
BF8	Not stated	Yes	Yusufeli, Artvin
BF9	Not stated	Yes	Yusufeli, Artvin
BF10	October 2005	Yes	Yusufeli, Artvin
BF11	October 2005	Yes	Yusufeli, Artvin
BF12	October 2005	Yes	Yusufeli, Artvin
BF13	June 2011	Yes	Çamlıhemşin, Rize
BF14	June 2011	Yes	Yusufeli, Artvin
BF15	October 2005	Yes	Yusufeli, Artvin

Table 2.2 (cont'd)

Sample ID	Date of Collection	Wild	Location
BF16	Not stated	yes	Yusufeli, Artvin
BF17	July 2011	yes	Şavşat, Artvin
BF18	July 2011	yes	Şavşat, Artvin
BF19	May 2010	yes	Yusufeli, Artvin
BF20	July 2011	yes	Şavşat, Artvin
BF21	July 2011	yes	Şavşat, Artvin
BF22	November 2011	no	Unknown (From Konya Zoo)
BF23	November 2011	no	Unknown (From Konya Zoo)
BF24	November 2011	no	Unknown (From Konya Zoo)
BF25	November 2011	no	Uludağ, Bursa (From Konya Zoo)
BF26	November 2011	yes	Akseki, Antalya
BF27	December 2011	no	Unknown
BF28	December 2011	no	Unknown
BF29	December 2011	no	Unknown (Hakkari/ Sivas/ Siirt)
BF30	December 2011	no	Unknown (Hakkari/ Sivas/ Siirt)
BF31	December 2011	no	Unknown (Hakkari/ Sivas/ Siirt)
BF32	December 2011	no	Unknown (Rize /Trabzon / Kastamonu)
BF33	December 2011	no	Unknown (Rize /Trabzon / Kastamonu)
BF34	December 2011	no	Unknown (Rize /Trabzon / Kastamonu)
BF35	December 2011	no	Unknown (Rize /Trabzon / Kastamonu)
BF36	December 2011	no	Unknown (Rize /Trabzon / Kastamonu)
BF37	December 2011	no	Unknown (Rize /Trabzon / Kastamonu)
BF38	December 2011	no	Unknown (Mixed: Kastamonu, Kocaeli)
BF39	December 2011	no	Unknown (Mixed: Kastamonu, Kocaeli)
BF40	December 2011	no	Unknown (Mixed: Kastamonu, Kocaeli)
BF41	December 2011	no	Unknown (Mixed: Kastamonu, Kocaeli)
BF42	December 2011	no	Unknown (Mixed: Kastamonu, Kocaeli)
BF43	December 2011	no	Unknown (Bursa Zoo)

Table 2.2 (cont'd)

BF44	December 2011	no	Unknown
BF45	December 2011	no	Unknown (from Antalya Zoo)
BF46	September 2011	yes	Yusufeli, Artvin
BF47	September 2011	yes	Yusufeli, Artvin
BF48	September 2011	yes	Yusufeli, Artvin
BF49	September 2011	yes	Yusufeli, Artvin

Fresh tissue samples were preserved in 95% ethanol, whereas old tissue samples were preserved in dry envelopes such like hair samples, until the time of DNA extraction. Sample IDs for both types of tissues, BT1-BT10 identification/enumeration was followed (Table 2.3).

Table 2.3: Old and fresh tissue sample IDs, date of collections, age of samples and the locations were shown

Sample ID	Date of Collection	Age of the Sample	Location
BT1	October 2011	Fresh	Yusufeli, Artvin
BT2	June 2011	Fresh	Yusufeli, Artvin
BT3	September 2010	Fresh	Yusufeli, Artvin
BT4	October 2010	Fresh	Yusufeli, Artvin
BT5	May 2010	Fresh	Yusufeli, Artvin
BT6	November 2011	>20 years	Borçka, Artvin
BT7	November 2011	>20 years	Akseki, Antalya
BT8	January 2012	10 years	Yusufeli, Artvin
BT9	December 2011	>15 years	Merkez, Artvin
BT10	January 2012	>10 years	İnegöl, Bursa
BT11	March 2012	>10 years	Kastamonu
BT12	March 2012	>10 years	Bilecik
BT13	July 2011	>20 years	İnegöl, Bursa
BT14	June 2011	>30 years	Şenkaya, Erzurum

The distribution of samples throughout Turkey can be seen in the locality map below (Figure 2.1).

Figure 2.1: Distribution of all type of samples throughout Turkey was shown. Pink, light blue, purple and yellow colours represent faeces, old tissue, fresh tissue and hair samples, respectively.

2.2 DNA Isolation and PCR

2.2.1 DNA extraction from hair

Prior to DNA extraction from hair samples, hair fibers with almost the same color and with fresh looking bulbs were selected under the microscope. It was aimed to collect more than 10 fibers for each extraction if applicable. After the selection, hair was washed in distilled water, 70% ethanol and in distilled water again, and this was repeated twice per each hair tuft. After the washing step, roots of the hair shafts were cut about 2-3mm long and used in the following lysis step.

For the DNA extraction from hair, Qiagen DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) manufacturer's instructions were followed with slight modifications. These modifications were basically in the lysis step:

- Washed and cut hair was placed in eppendorf tubes with 180µl ATL Buffer, 20 µl 0.15M DDT, 20 µl 20 mg/mL Proteinase K

- Eppendorf tubes were placed in the 65^{o}C water bath and they were vortexed regularly until the bulbs disappeared (overnight or at most two nights long incubation).

After obtaining extracts following the steps mentioned above, the protocol for Purification of Total DNA from Animal Tissues (Spin-Column Protocol) was followed from step 3.

- Each extract in the properly labeled eppendorf tubes was vortexed for 15s. 200 µl Buffer AL added and the tubes were mixed thoroughly by vortexing. Afterwards, 200 µl of absolute ethanol was added and mixed thoroughly by vortexing.

- The mixtures, including any precipitates in the previous step were pipetted into the DNeasy Mini spin columns that were placed in 2 ml collection tubes provided in the kit. Then spin columns were centrifuged at 8000 rpm for 1 min. Flow-throughs and the collection tubes were discarded.

DNeasy Mini spin columns were placed in new 2 ml collection tubes and 500 µl Buffer AW1 was added on them. Then spin columns were centrifuged for 1 min. at 10000 rpm and flow-throughs and collection tubes were discarded again.

- DNeasy Mini spin columns were placed in new 2 ml collection tubes and 500 µl Buffer AW2 was added on them. Then spin columns were centrifuged for 3 min. at 14000 rpm and flow-throughs and collection tubes were discarded again.

- DNeasy Mini spin columns were placed in new 1.5 ml eppendorf tubes and 100 µl of AE Buffer was added directly onto the DNeasy membranes. All tubes were incubated at room temperature for 1 min and then they were centrifuged at 12000 rpm for 1 min.

After the elution step of the DNA, extracts were placed at +4^{o}C until the time of absorbance measurement. For the absorbance measurement, 1 µl per each

extract was used and the measurements were done with Thermo Scientific NanoDrop 2000 Spectrophotometer. Concentration and purity of DNA was determined by the readings at 260 nm and 280 nm.

For long term storage of the isolates, samples were stored at -20^{o}C.

2.2.2 DNA extraction from faeces

There are five different methods applied in this study during DNA extraction from faeces. Three of them are procedures of different commercial stool DNA isolation kits (Qiagen QIAamp DNA Stool Mini Kit, Zymo Research Fecal DNA MiniPrep™, NORGEN Stool DNA Isolation Kit) and two of them are kit procedures applied with modifications (GuSCN (Buffer L6) + Qiagen QIAamp DNA Stool Mini Kit and GuSCN (Buffer L6) + Qiagen DNeasy Blood and Tissue Kit).

For each method, the outer parts of the faeces were scraped away and the scrapings were used for the lysis steps.

Qiagen QIAamp DNA Stool Mini Kit Protocol: Isolation of DNA from Stool for Human DNA Analysis

- 180-220 mg of stool was weighed and placed in each properly labeled 2 ml microcentrifuge tubes (eppendorf tubes).
- 1.6 ml Buffer ASL was added to each stool sample and all samples were vortexed until the stools become completely homogenized.
- Samples were centrifuged at full speed (14000 rpm) for 1 min. to pellet stool particles.
- 1.4 ml of the supernatant was pipetted into new 2 ml microcentrifuge tubes and pellets were discarded.
- 1 InhibitEX Tablet was added into each tube and the tubes were vortexed immediately and continuously for 1 min. or until the tablet is completely suspended. Suspensions were incubated for 1 min at room

temperature in order to allow inhibitors to adsorb to the InhibitEX matrix.

- All samples were centrifuged at full speed for 3 min to pellet stool particles and the inhibitors bound to InhibitEX matrix.
- Immediately after the centrifuge had stopped, all of the supernatants pipetted into new 1.5 ml microcentrifuge tubes that were properly labeled. The pellets were discarded and all samples were centrifuged for an additional 3 min.
- 25 µl of 20 mg/ml proteinase K was pipetted into new 2 ml microcentrifuge tubes.
- 600 µl supernatant pipetted from the centrifuged tubes to the 2 ml microcentrifuge tubes containing proteinase K.
- 600 µl Buffer AL was added to each tube and all the tubes were vortexed for 15s.
- Samples were incubated at 70^{o}C for 10 min.
- 600 µl of cold absolute ethanol was added into each lysate and the tubes were mixed by vortexing.
- Lids of QIAamp spin columns provided with the kit were labeled and placed on 2 ml collection tubes. 600 µl of lysates obtained from the previous step were applied onto the spin columns without moistening the rim. Caps were closed and tubes were centrifuged at full speed for 1 min. After centrifugation spin columns were placed in new 2 ml collection tubes and tubes containing with filtrates were discarded.
- QIAamp spin columns were carefully opened and the second aliquot of 600 µl lysates were applied and centrifuged at full speed for 1 min. Spin columns were placed in new 2 ml collection tubes and the tubes containing filtrates were discarded.
- Previous step was repeated to load the third aliquot of the lysate onto the spin columns.

- Spin columns were carefully opened and 500 µl Buffer AW1 was added. Tubes were centrifuged at full speed for 1 min. Collection tubes containing filtrates were discarded.

- Spin columns were carefully opened and 500 µl Buffer AW2 was added. Tubes were centrifuged at full speed for 3 min. Collection tubes containing filtrates were discarded.

- Spin columns were placed in new 2 ml collection tubes and centrifuged at full speed for 1 min. in order to eliminate the residual Buffer AW2.

- Spin columns were transferred into new labeled 1.5 ml microcentrifuge tubes. 50-70 µl of Buffer AE was pipetted and applied directly onto the QIAamp membrane. The tubes were incubated for 1 min. at room temperature, then centrifuged at full speed for 1 min. to elute DNA.

Zymo Research Fecal DNA MiniPrep™ protocol:

- Before starting: Zymo-Spin IV-HRC Spin filters (green tops) need to be prepared prior to use by: 1) Snapping off the base 2) inserting into a Collection Tube and 3) spinning in a microcentrifuge at exactly 11000 rpm for 3 min.

- Up to 150 mg of fecal samples were added to ZR BahingBead Lysis Tube and 750 µl Lysis Solution added to the tubes.

- ZR BashingBead Lysis Tubes were centrifuged at 12100 rpm for 1 min.

- Up to 400 µl supernatant was transferred to Zymo-Spin IV Spin Filter (orange top) in Collection Tubes and they were centrifuged at 10300 rpm for 1 min.

- 1.2 ml of Fecal DNA Binding Buffer was added into filtrates in the Collection tubes in the previous step.

- 800 µl of the mixture from the previous step was transferred to a Zymo-Spin IIC Columns in Collection Tubes and tubes were centrifuged at 12500 rpm for 1 min. Flow throughs were discarded.

- Previous step was repeated.

- 200 µl of DNA Pre-Wash Buffer was added to the Zymo-Spin IIC Column in new Collection Tubes and the tubes were centrifuged at 12500 rpm for 1 min.
- 500 µl of Fecal DNA Wash Buffer was added to the Zymo-Spin IIC Column and the tubes were centrifuged at 12500 rpm for 1 min.
- Zymo-Spin IIC Columns were transferred to clean 1.5 ml microcentrifuge tubes and 25-100 µl of DNA Elution Buffer was added directly onto the column matrices. The tubes were centrifuged at 12300rpm for 30 seconds in order to elute the DNA.
- Eluted DNA was transferred to previously prepared Zymo-Spin IV-HRC Spin Filter (green top) in clean 1.5 microcentrifuge tubes and the tubes were centrifuged at 11100 rpm for 1 min. The eluted and filtered DNA was then prepared for PCR and other downstream applications.

NORGEN Stool DNA Isolation Kit Procedures:

- Up to 200 mg of stool samples were added into provided Bead Tubes and 1mL of Lysis Solution was added into the tubes. In order to mix stool and Lysis Solution, the tubes were vortexed briefly (until the mixture became homogenized).
- 100 µl of Lysis Additive was added and the tubes were vortexed briefly.
- When Lysis Additive seemed to be suspended, the tubes were vortexed for additional 5 min.
- The tubes were centrifuged for 2 min. at 14000 rpm.
- Up to 600 µl of supernatant transferred to a DNase-free microcentrifuge tubes.
- 200 µl of Binding Solution was added and the tubes were mixed by inverting the tubes few times and they were incubated on ice for 10 min.
- Lysates were span for 2 min to pellet any cell debris.

- Up to 700 µl of supernatant transferred by avoiding contact with the pellet into new 2ml DNase-free microcentrifuge tubes.
- Equal volume of 70% ethanol was added onto each lysate collected above. Tubes were vortexed to be mixed.
- Spin columns were assembled with the provided collection tubes.
- 600 µl of the clarified lysate with ethanol was applied onto each column and they were centrifuged for 1 min at 14000 rpm. Flow-throughs were discarded and the spin columns were reassembled with the collection tubes.
- Previous step was repeated until the all lysate was filtered through spin columns.
- 500 µl of Wash Solution I was applied to the columns and the tubes were centrifuged for 1 min.
- Flow-throughs were discarded and the spin columns were reassembled with their collection tubes.
- 500 µl of Wash Solution II was applied to the columns and the tubes were centrifuged for 1 min.
- Flow-throughs were discarded and the spin columns were reassembled with their collection tubes.
- Washing step with Wash Solution II was repeated.
- The columns were span for 2 min. in order to thoroughly dry the resin. Collection tubes were discarded.
- Columns were placed into fresh 1.7 ml Elution tubes provided with the kit.
- 50 µl of Elution Buffer was added onto the columns.
- Tubes were firstly centrifuged for 2 min at 2000 rpm, and then 1 additional min. of centrifugation at 14000rpm was followed.

For the modified kit procedures, initially GuSCN containing L6 Buffer (10 M GuSCN, 0.1 M Tris-HCl pH 6.4, 0.02 M EDTA pH8.0, 1.3% Triton X-100) was prepared (see Boom *et al.*, 1990 and Taberlet *et al.*, 1997). Scraped particles from fecal samples were added into 2 ml microcentrifuge tubes and L6 Buffer was added onto the samples until the tubes were filled. Tubes were vortexed regularly and placed into water bath at 60°C for a proper lysis until the fecal samples were homogenized in the tubes (from overnight to 2 days incubation). After fecal samples were homogenized tubes were centrifuged at 14000 rpm for 3 min. By avoiding contact with cell debris, all supernatant was pipetted and transferred to 2 ml DNase-free microcentrifuge tubes.

For the GuSCN (Buffer L6) + Qiagen QIAamp DNA Stool Mini Kit Procedure, lysis step was applied as explained above. 1.6 ml of ASL Buffer was added into new 2 ml microcentrifuge tubes and the transferred supernatant was added into the tubes containing ASL Buffer, until the tube was filled. All tubes were centrifuged for 1 min. and the following steps were the identical with the steps of the Qiagen QIAamp DNA Stool Mini Kit Procedure.

For the GuSCN (Buffer L6) + Qiagen DNeasy Blood and Tissue Kit Procedure, 20 µl of 20 mg/ml proteinase K was added onto the collected supernatants. The tubes were incubated at 56°C for 10-15 min in the water bath, and the same volume of Buffer AL was added and tubes were vortexed for 15s. Then again the same volume of ice cold absolute ethanol was added to the mixture and tubes were mixed with vortexing. Afterwards, up to 750 µl of the mixture was transferred to the DNeasy Mini Spin column placed in a 2 ml collection tubes. The tubes were centrifuged at 8000 rpm for 1 min. and the flow-throughs were discarded. Filtration through spin columns was repeated until the all collected supernatant, Buffer AL and ethanol mixture was transferred. Washing steps and the elution step was identical of the previously mentioned Qiagen DNeasy Blood and Tissue Kit procedure.

Table 2.4: Five distinct fecal DNA isolation procedures and the samples that were applied with them were shown.

DNA Extraction Procedure	Samples
Qiagen QIAamp Stool Kit	BF1-6; BF15-16; BF22-25
Zymo Research Fecal DNA MiniPrep™	BF7-12
NORGEN Stool DNA Isolation Kit	BF6; BF13; BF25-26; BF29-38; BF45-49
GuSCN + Qiagen QIAamp Stool Kit	BF4-6;BF8-9;BF13-21
GuSCN +Qiagen DNeasy Blood and Tissue	BF7; BF12; BF20-21; BF26-44

After the DNA elution step, extracts were placed at +4°C until the time of absorbance measurement. For the absorbance measurement, 1 µl per each extract was used and the measurements were done with Thermo Scientific NanoDrop 2000 Spectrophotometer. Concentration and purity of DNA was determined by the readings at 260 nm and 280 nm. For long term storage of the isolates, samples were stored at -20°C.

2.2.3 DNA extraction from tissue samples

Procedures followed for old and fresh tissue DNA extractions were separate. Fresh tissue samples were washed twice with distilled water and 70% ethanol before the lysis step. For all fresh tissue samples, samples were first cut into pieces and crushed with sand until pulverized. Then, lysates were applied either with Qiagen DNeasy Blood and Tissue Kit or Phenol/Chloroform DNA Isolation. For the samples used in Qiagen DNeasy Blood and Tissue Kit, manufacturer's instructions of Purification of Total DNA from Animal Tissues (Spin-Column Protocol) were followed.

The procedure of Phenol/ Chloroform DNA Isolation applied on other fresh tissue samples:

- 180 µl ATL Buffer and 20 µl 20 mg/ml proteinase K was added onto the lysate in the microcentrifuge tubes. The tubes were vortexed regularly and the tubes were incubated overnight at 56°C

- 1:1 (approximately 750 µl) phenol was added in the tubes. Tubes were inverted few times and centrifuged at 13800 rpm for 17 min.
- After centrifugation, 530 µl of supernatant was transferred to clean 1.5 ml microcentrifuge tubes.
- 1:1 (530 µl) phenol was added to clean tube; phenol and lysate was inverted several times for mixing. Tubes were centrifuged at 13800 rpm for 17 min.
- 300 µl of supernatant was transferred to 1.5 ml clean microcentrifuge tubes and 210 µl isopropanol was added. Tubes were inverted again, and centrifuged for 3 min. at 13800 rpm.
- Supernatant was carefully removed and 1ml of ice cold absolute ethanol was added onto them.
- The tubes were kept at -20^{o}C for 10 min.
- Tubes were centrifuged at 13800 rpm for 5 min.
- Ethanol was removed and the pellets were dried out.
- On the dried pellets, 500 µl of TE- Acetate-RNAse Buffer was added and the pellets were suspended in the buffer by tapping and incubating at 60^{o}C
- When pellets were seemed to be dissolved, water bath was set to 37^{o}C and tubes were incubated overnight.
- After incubation 1ml of ice cold absolute ethanol was added. Samples were inverted several times and incubated at -20^{o}C for 2 hours.
- After 2 hours, all samples were centrifuged at 13800 rpm for 5 min.
- Supernatant was discarded from all the tubes and 1 ml of 70% ethanol was added. Tubes were inverted several times.
- All samples were centrifuged at 13800 rpm for 5 min.
- Last two steps were repeated again.

- Ethanol was removed and the pellets were completely dried out.
- 100 µl TE Buffer was added into sampled and the pellets were suspended by tapping and incubating at +60°C.

Old tissue samples were washed twice with distilled water and 70% ethanol before the lysis step. For all old tissue samples, samples were first cut into pieces and crushed with liquid nitrogen until pulverized. Then, lysates were applied either with Qiagen DNeasy Blood and Tissue Kit, NORGEN Stool DNA Isolation Kit or GuSCN +Qiagen DNeasy Blood and Tissue Kit.

For the samples used in Qiagen DNeasy Blood and Tissue Kit, manufacturer's instructions of Purification of Total DNA from Animal Tissues (Spin-Column Protocol) were followed. For the samples used in NORGEN Stool DNA isolation Kit, 200 mg tissue lysate was used instead of 200 mg fecal sample. All other steps were identical as mentioned in the previous section. For the samples used in GuSCN + Qiagen DNeasy Blood and Tissue Kit, manufacturer's instructions of Purification of Total DNA from Animal Tissues (Spin-Column Protocol) were followed after the crash lysates incubated with L6 Buffer at +60°C overnight.

Table 2.5: Distinct DNA extraction methods applied for fresh and old tissue samples were shown. Old tissue samples were highlighted with asterisk.

DNA Extraction Procedure	Samples
Qiagen DNeasy Blood and Tissue Kit	BT13*, BT14*, BT15
NORGEN Stool DNA Isolation Kit	BT6*, BT7*, BT8*, BT9*, BT10*, BT11*, BT12*
GuSCN +Qiagen DNeasy Blood and Tissue Kit	BT6*, BT7*
Phenol / Chloroform DNA Isolation	BT1-BT5

After DNA isolates were obtained from each of extraction method, extracts were placed at +4°C until the time of absorbance measurement. For the absorbance measurement, 1 µl per each extract was used and the measurements were done with Thermo Scientific NanoDrop 2000 Spectrophotometer. Concentration

and purity of DNA was determined by the readings at 260 nm and 280 nm. For long term storage of the isolates, samples were stored at -20^{o}C.

2.2.4 DNA amplification

In order to amplify the 269 bp mtDNA control region, two distinct types of PCR were applied. For the first type, 2-10 µl of genomic DNA elutes and the primers 5'-CTCCACTATCAGCAC-CCAAAG-3' (forward) and 5'-GGAGCGAGAAGAGG-TACACGT-3' (reverse) (Taberlet & Bouvet 1994) were used for the amplification of 269 bp mtDNA control region. Reaction mixture was prepared with 2-10 µl genomic DNA, 5 µl of 10 x PCR Buffer, 2.5 µl of 25 mM $MgCl_2$, 10 pmols of each primer, 1 µl of 10 mM dNTP and 0.2 unit of Taq Polymerase and dH_2O was added until the total volume reaches to 25 µl. The PCR program for the amplification ran at 93^{o}C for 3 min, followed by 45 cycles of 93^{o}C for 1 min, 50^{o}C for 1 min and 72^{o}C for 1.5 min, and a final 5 min extension at 72^{o}C. For the samples giving weak outcomes with this primer set, nested PCR were performed using the primer set L15774 (Kocher *et al.*, 1989) and H16498 (Shields & Kocher, 1991) and amplified a 400 bp piece of mtDNA from the *cytochrome b* gene to the control region (Shields & Kocher, 1991). The weak PCR product obtained from this latter primer set was used as a template to amplify the 269 bp mtDNA control region and obtain a well product, this time using the primer set of Taberlet & Bouvet (1994).

Negative controls were included into each sample set in order to monitor contamination. For the monitoring, PCR products were run on 2% agarose gel was prepared in TAE Buffer and 7 µl ethidium bromide was added. Agarose gel electrophoresis was performed at 110 V for approximately 30 min. DNA bands were screened by Vilber Lourmat UV transilluminator and the band lengths were determined with the help of GeneRuler™ 100bp Plus DNA Ladder.

Finally, PCR products were purified by following the general procedure of GeneMark Gel Elution Kit:

- Total volume of PCR products were added directly onto the spin column membrane.

- Then, on each spin column membrane, 500 µl of Binding Solution was added.
- Spin columns were inserted into Collection tubes and the tubes were centrifuged at 14000 rpm for 1 min. The filtrate in the collection tubes was discarded.
- 700 µl of Washing Solution was added into the spin columns and the tubes were centrifuged at 14000 rpm for 1 min. Filtrates were discarded.
- The previous –washing step- was repeated.
- Tubes were centrifuged for 3-5 min. at 14000 rpm in order to remove residual trace of ethanol.
- Spin columns were transferred to new microcentrifuge tubes and 30 µl distilled water (pH7.0-8.5) was added directly onto the spin column membranes. Tubes were incubated at room temperature for 1-2 min.
- The tubes were centrifuged at 14000 rpm for 1 min for DNA elution. Eluted DNA in microcentrifuge tubes was stored at -20^{o}C until the time of analysis.

2.3 DNA sequencing and alignment

ABI terminator 3.1 Kit (Applied Biosystems Inc., Foster City, CA, USA) executed all sequencing reactions. Sequencing of PCR products was performed in forward and reverse directions in order to increase accuracy. Electrophoresis and detection of fluorescently labeled nucleotides were made with an automatic DNA sequencer (ABI 3730x1 Genetic Analyzer, Applied Biosystems). Mitochondrial DNA sequences representing distinct haplotypes were recorded in order to be deposited at GenBank.

MEGA 5.1 (Tamura *et al.*, 2011) including CLUSTAL W algorithm was performed for the alignment of sequences. Bayesian phylogenetic analysis, network construction, genetic diversity index and genetic distance calculations were performed with the 269 bp long alignment dataset.

Determination of Sample Success

Sample success was defined as the percentage of samples out of all samples from which reliable sequence for alignment was obtained.

2.4 Data Analysis

2.4.1 Genetic diversity

ARLEQUIN 3.5 (Excoffier & Lischer, 2010) was used for the calculation of haplotype frequency and diversity (h) as well as nucleotide diversity (π) (Nei, 1987). In order to calculate molecular diversity indices for particular clades and the subclades found in Turkey, and to compare them with previously defined such clades, haplotype diversity and nucleotide diversity calculations were done. For this analysis, the groups which have sample sizes larger than 5 were used.

Uncorrected p distances were calculated in MEGA 5.1 (Tamura *et al.*, 2011) to define boundaries of clades, subclades, and populations by following the equation $p = n_d / L$, where n_d stands for the number of transitions + transversion and L refers for the total number of valid common sites.

2.4.2 Phylogenetic analysis

To evaluate the phylogenetic position of Anatolian brown bear populations within brown bears of the Western Palearctic, 63 distinct mtDNA control region haplotypes (total of 79 sequences) corresponding to maternal lineages identified from Western and Eastern Europe, Middle East, Inner Asia and North Africa were downloaded from GeneBank and combined with the haplotypes revealed in this study, whereas American black bear (*Ursus americanus*) and cave bear (*Ursus spelaeus*) sequences were used as outgroups (Table 2.6).

Table 2.6: Downloaded mtDNA sequences used in the alignment dataset of this study is shown. First two letters of Sample IDs were constructed due to the location where samples were taken; abbreviations are the country codes top-level domain. Samples with three letter ID corresponds to the samples taken from zoos (i.e. IR-Z1 etc.) Samples with IDs starting with XX correspond to unknown origin.

Sample IDs in this study	Accession No.	Location	Species	Reference
GE-1	GU057343	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-2	GU057345	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-3	GU057346	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-4	GU057347	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-5	GU057349	Georgia-Greater Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-6	GU057351	Georgia-Greater Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-7	GU057352	Georgia-Greater Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-8	GU057353	Georgia-Greater Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-9	GU057356	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-10	GU057357	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-11	GU057358	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-12	GU057359	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-13	GU057363	Georgia-Greater Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-14	GU057366	Georgia-Greater Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-15	GU057367	Georgia-Greater Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-16	GU057368	Georgia-Greater Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-17	GU057369	Georgia-Greater Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010

Table 2.6 (cont'd)

Sample IDs in this study	Accession No.	Location	Species	Reference
GE-18	GU057371	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-19	GU057372	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-20	GU057373	Georgia-Greater Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-21	GU057374	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-22	GU057375	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
GE-23	GU057376	Georgia-Lesser Caucasus	*U. arctos*	Murtskhvaladze *et al.* 2010
SK-1	X75876	Slovakia	*U. arctos*	Taberlet & Bouvet 1994
GR-1	X75870	Greece	*U. arctos*	Taberlet & Bouvet 1994
BA-1	X75877	Bosnia	*U. arctos*	Taberlet & Bouvet 1994
HR-1	X75867	Croatia	*U. arctos*	Taberlet & Bouvet 1994
BG-1	X75864	Bulgaria	*U. arctos*	Taberlet & Bouvet 1994
FR-1	X75878	France	*U. arctos*	Taberlet & Bouvet 1994
SE-1	X75868	Sweden	*U. arctos*	Taberlet & Bouvet 1994
RO-1	X75872	Romania	*U. arctos*	Taberlet & Bouvet 1994
ES-1	X75865	Spain	*U. arctos*	Taberlet & Bouvet 1994
RO-2	X75873	Romania	*U. arctos*	Taberlet & Bouvet 1994
CN-1	X75863	Tibet	*U. arctos*	Taberlet & Bouvet 1994
AT-1	FN663157	Austria	*U.spelaeus*	Stiller *et al.* 2010

Table 2.6 (cont'd)

Sample IDs in this study	Accession No.	Location	Species	Reference
HR-2	HQ602653	Croatia	*U. arctos*	Kocijan *et al.* 2011
HR-3	HQ602652	Croatia	*U. arctos*	Kocijan *et al.* 2011
HR-4	HQ602651	Croatia	*U. arctos*	Kocijan *et al.* 2011
XX-Z1	FN292981	Unknown Origin-Heidelberg Zoo	*U. arctos*	Calvignac *et al.* 2009
XX-Z2	FN292980	Unknown Origin-Heidelberg Zoo	*U. arctos*	Calvignac *et al.* 2009
XX-Z3	FN292979	Unknown Origin-Montpelier Zoo	*U. arctos*	Calvignac *et al.* 2009
XX-Z4	FN292978	Unknown Origin-Montpelier Zoo	*U. arctos*	Calvignac *et al.* 2009
XX-Z5	FN292982	Unknown Origin - Ostrava Zoo	*U. arctos*	Calvignac *et al.* 2009
IR-Z1	FN292977	Paris Zoo	*U. arctos*	Calvignac *et al.* 2009
IR-Z2	FN292976	Paris Zoo	*U. arctos*	Calvignac *et al.* 2009
IR-1	FN292974	Iran	*U. arctos*	Calvignac *et al.* 2009
IR-2	FN292975	Iran	*U. arctos*	Calvignac *et al.* 2009
SY-1	FN292973	Syria	*U. arctos*	Calvignac *et al.* 2009
LB-1	FN292972	Lebanon	*U. arctos*	Calvignac *et al.* 2009
LB-2	FN292971	Lebanon	*U. arctos*	Calvignac *et al.* 2009
LB-3	FN292970	Lebanon	*U. arctos*	Calvignac *et al.* 2009
RU-1	EU526794	Siberia, Russia	*U. arctos*	Korsten *et al.* 2009
US-1	EF198825	USA	*U.americanus*	Robinson *et al.* 2007

Table 2.6 (cont'd)

CN-2	AB010727	Tibet	*U. arctos*	Masuda *et al.* 1998
MN-1	AB010728	Gobi	*U. arctos*	Masuda *et al.* 1998
MA-1	AM411399	Morocco	*U. arctos*	Calvignac *et al.* 2008
DZ-1	AM411400	Algeria	*U. arctos*	Calvignac *et al.* 2008
CN-3	DQ914407	Tibet	*U. arctos*	Miller *et al.* 2006
IR-3	DQ914408	Iran	*U. arctos*	Miller *et al.* 2006
PK-1	DQ914409	Pakistan	*U. arctos*	Miller *et al.* 2006
PK-2	DQ914410	Pakistan	*U. arctos*	Miller *et al.* 2006
XX-Z6	DQ914411	Unknown Origin- Greek Zoo	*U. arctos*	Miller *et al.* 2006
RO-3	L38270	Romania	*U. arctos*	Kohn *et al.* 1995
RO-4	L38272	Romania	*U. arctos*	Kohn *et al.* 1995
ES-2	EF488487	Spain	*U. arctos*	Valdiosera *et al.* 2007
ES-3	EF488503	Spain	*U. arctos*	Valdiosera *et al.* 2007
FR-2	EF488495	France	*U. arctos*	Valdiosera *et al.* 2007
ES-4	EF488504	Spain	*U. arctos*	Valdiosera *et al.* 2007
IT-1	EF488488	Italy	*U. arctos*	Valdiosera *et al.* 2007
ES-5	EF488490	Spain	*U. arctos*	Valdiosera *et al.* 2007
FR-3	EF488496	France	*U. arctos*	Valdiosera *et al.* 2007
FR-4	EF488492	France	*U. arctos*	Valdiosera *et al.* 2007
FR-5	EF488493	France	*U. arctos*	Valdiosera *et al.* 2007
FR-6	EF488491	France	*U. arctos*	Valdiosera *et al.* 2007
FR-7	EF488494	France	*U. arctos*	Valdiosera *et al.* 2007
FR-8	EF488505	France	*U. arctos*	Valdiosera *et al.* 2007
ES-6	EF488497	Spain	*U. arctos*	Valdiosera *et al.* 2007
DE-1	EF488501	Germany	*U. arctos*	Valdiosera *et al.* 2007
DE-2	EF488498	Germany	*U. arctos*	Valdiosera *et al.* 2007
DE-3	EF488499	Germany	*U. arctos*	Valdiosera *et al.* 2007
AT-2	AJ809334	Austria	*U. arctos*	Hofreiter *et al.* 2004

2.4.2.1 Model of DNA sequence evolution

The appropriate model for nucleotide substitution was determined by jModeltest (Posada, 2008) which applies five distinct model selection strategies such as hierarchical and dynamical likelihood ratio tests (hLRT and dLRT), Akaike and Bayesian information criteria (AIC and BIC), and a decision theory method (DT). The best probabilistic model of sequence evolution was determined by considering Akaike and Bayesian Information Criteria.

2.4.2.2 Construction of phylogenetic tree and calculation of divergence times

The program BEAST 1.7.1 (Drummond *et al.*, 2012) was used for the analysis of phylogeny and divergence times. The dataset was prepared for the analysis by BEAUTI 1.7.1 (Drummond *et al.*, 2012).

In this study two phylogenetic analyses were performed: one with mtDNA control region sequences that were only 193 bp long, and one with sequences from the same region that were 269 bp long. The dataset with 193 bp long sequences contains 21 ancestral sequences that included some short sequences. For these 21 ancestral sequences, the carbon-date in years was used as the sampling date. The longer dataset (269 bp) contained only contemporary samples, except for *U. spelaeus*.

Lognormal priors with two parameters, the mean rate and a standard deviation, were used in order to estimate a relaxed molecular clock calibration (Drummond *et al.*, 2006). For the rate mean, a narrow normal distributed hyperprior with a mean of 0.39 per million years and a standard deviation of 0.08 per million years were used because of the short sequences in the dataset (Ho *et al.* 2008). Also, a normal distributed hyperprior for the standard deviation parameter of the lognormal distribution with mean of 0.08 and standard deviation of the same magnitude were used. Priors for the mutation model were set as default. Finally, the majority consensus tree of the 269 bp dataset, generated with sumtrees.py (Sukumaran & Holder 2010), and the timings from the maximum posterior of the dataset with the dated samples (TREEANNOTATOR 1.7.1, Drummond *et al.* 2012) were reported.

2.4.2.3 Median joining network

A median-joining network was constructed with the software NETWORK version 4.6.1.0 (Bandelt *et al.*, 1999) in order to deduce evolutionary relationships and probable ancestral connections among different haplotypes. In the construction of network, only sequences of 269 bp length were used and shorter sequences removed from the dataset to avoid the loss of information.

CHAPTER 3

RESULTS

3.1 DNA Isolation and PCR

3.1.1 DNA isolation from distinct types of DNA sources

From each of the 49 faeces samples, DNA was managed to be isolated. As mentioned previously, there are 5 different DNA isolation procedures followed in this study and the mean DNA concentrations revealed from each procedure are summarized in Table 3.1.

Table 3.1: Comparison of 5 different faecal DNA extraction procedures by means of average amount of isolated DNA.

DNA Extraction Procedure	Number of Trials / Replicates	Average Concentration of Isolated DNA (ng/µl)
Qiagen QIAamp Stool Kit	12	230.00
Zymo Research Fecal DNA MiniPrep™	4	59.55
NORGEN Stool DNA Isolation Kit	15	243.05
GuSCN + Qiagen QIAamp Stool Kit	28	277.55
GuSCN +Qiagen DNeasy Blood and Tissue Kit	32	458.13
Overall	**91**	**319,51**

Table 3.1 shows that the largest amount of DNA was revealed with GuSCN + Qiagen DNeasy Blood and Tissue Kit and then followed by GuSCN+Qiagen

QIAamp Stool Kit, NORGEN Stool DNA Isolation Kit, Qiagen QIAamp Stool Kit and Zymo Research Fecal DNA MiniPrep™, respectively.

From 6 of the total 47 hair samples, DNA was not managed to be isolated. Because, all samples were treated with the same procedure (DDT+DNeasy Blood and Tissue Kit); average concentration of isolated DNA from 41 samples was calculated as 4,55 ng/µl.

Total of 15 tissue samples (9 old, 6 fresh tissue) were treated and none of them failed in DNA isolation step. There were 4 different DNA extraction procedures followed in this study and the comparison of them are summarized in Table 3.2 shown below.

Table 3.2: Comparison of 4 different tissue DNA extraction procedures by means of average amount of isolated DNA. Treatments applied to old tissue samples are highlighted with asterisk.

DNA Extraction Procedure	**Number of Trials / Replicates**	**Average Concentration of Isolated DNA (ng/µl)**
Qiagen DNeasy Blood and Tissue Kit	2	66.25
NORGEN Stool DNA Isolation Kit*	6	6.33
GuSCN +Qiagen DNeasy Blood and Tissue Kit*	10	56.64
Phenol / Chloroform DNA Isolation	5	48.12

When fresh and old tissue averages were compared, it can be seen that 7 trials gave the average of 53.30 ng/µl DNA whereas 16 trials of old tissue samples' average was 37.77 ng/µl.

To sum up, the largest average amount of isolated DNA was from faecal samples then fresh tissue, old tissue and hair samples followed, respectively.

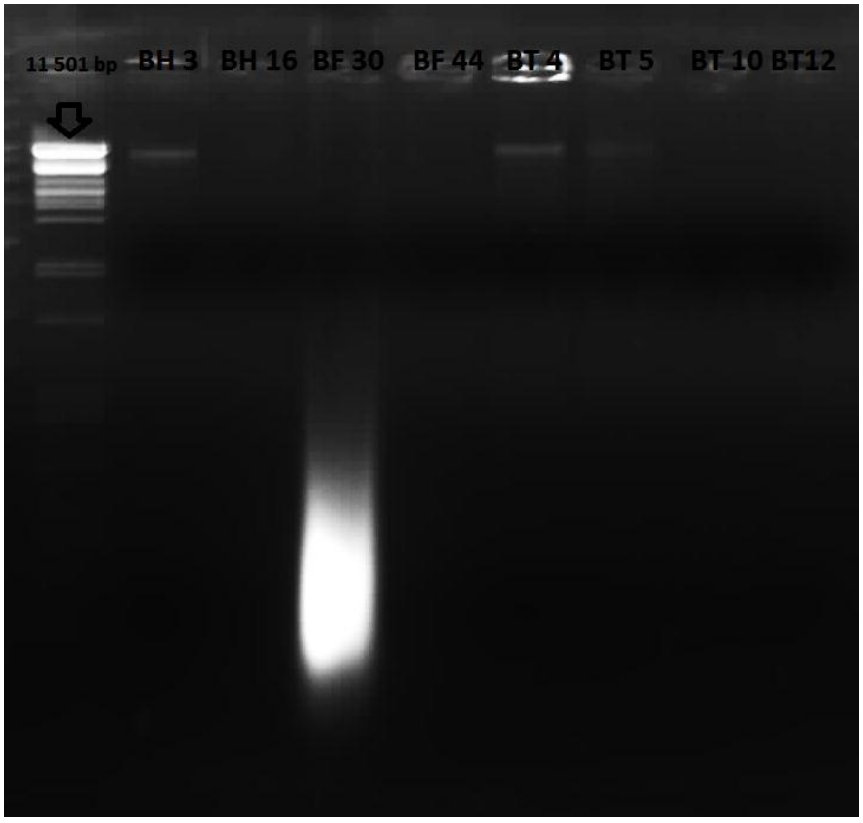

Figure 3.1: Genomic DNA gel photograph of two hair (BH13, 16); faeces (BF30,44); fresh tissue (BT4,5) and old tissue samples (BT10,12) was shown.

BH3, BT4 and BT5 gave thin bands located on the 11,501bp of the marker whereas no bands can be seen for BH16 BF44, BT10 and BT12 indicating a trace amount of DNA.

The shiny area seen on the BF30 column can be the result of inhibitors in the DNA extract that comes from a faecal material.

Table 3.3: Comparison of average concentration of isolated DNA from distinct types of DNA sources. Superscripts denote results significantly different from others.

Sample Type	Average Concentration of Isolated DNA
Hair	4.55 ^{a}
Faeces	319.51^{b}
Old Tissue	37.77 ^{c}
Fresh Tissue	53.30 ^{c}

Table 3.3 shows that the average concentration of isolated DNA from different sample sources. DNA concentrations from both hair and faeces significantly differ from all other sample sources at $p<0.01$.

3.1.2 PCR and nested PCR

Sample of PCR results can be seen from the figure below:

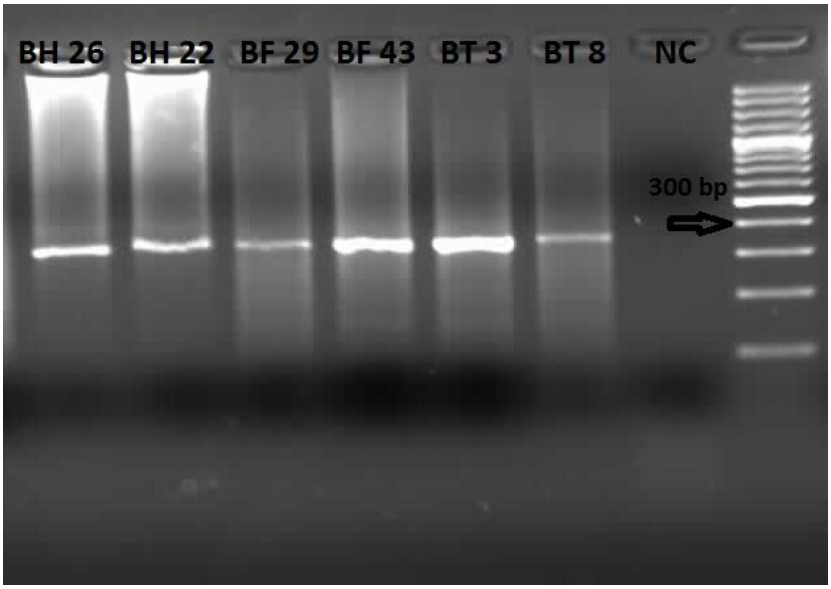

Figure 3.2: Products of polymerase chain reaction conducted with the primer set used in Taberlet & Bouvet (1994) and applied for the samples BH26, 22; BF29, 43; BT3, 8 and negative control were shown.

In figure 3.2 DNA bands of the PCR products of all sample types were observed slightly below the 300bp line of the marker pointing out the 269bp mtDNA control region amplification.

Calculation of sample success was done as mentioned before. Success percentages belonging to distinct types of DNA sources are shown in Table 3.4. Also, for comparison, success percentages belonging to different faecal DNA extraction methods are shown in Table 3.5.

Table 3.4: Success percentages of the samples isolated from four different DNA sources.

DNA Source	% DNA Extraction Success	% PCR Success	% Sample Success
Hair	87.23	38.77	34.69
Faeces	100.00	40.81	30.61
Old Tissue	100.00	44.44	22.22
Fresh Tissue	100.00	83.30	83.30

Table 3.5: Success percentages of five different faecal DNA extraction procedures

Faecal DNA Extraction Procedure	% Success
Qiagen QIAamp Stool Kit	20.00
Zymo Research Fecal DNA MiniPrep™	13.33
NORGEN Stool DNA Isolation Kit	20.00
GuSCN + Qiagen QIAamp Stool Kit	13.33
GuSCN +Qiagen DNeasy Blood and Tissue Kit	33.33

All in all, from 13 faecal, 5 fresh tissue, 2 old tissue and 15 hair samples reliable sequences were obtained and these were added to the DNA Alignment dataset. However, in order to ease the appearance in data analysis processes Sample IDs of these samples were converted as shown in Table 3.6.

Table 3.6: Sample ID conversion of successful samples in PCR and DNA sequencing. Sample IDs including –Z1-5 represent 6 captive individuals.

New Sample ID	Old Sample ID
TR-1	BT8
TR-2	BH26
TR-3	BH22
TR-4	BH29
TR-5	BH46
TR-Z1	BF29
TR-6	BT1
TR-7	BT3
TR-8	BT4
TR-9	BF8
TR-10	BF9
TR-11	BH31
TR-12	BH8
TR-13	BH9
TR-14	BH47
TR-15	BH39
TR-16	BH34
TR-17	BT5
TR-18	BH5
TR-19	BF21
TR-20	BH15
TR-21	BH6
TR-22	BF7
TR-Z3	BF43
TR-Z4	BF44
TR-23	BT7
TR-24	BF26

Table 3.6 (cont'd)

TR-Z6	BF25
TR-25	BH3
TR-26	BF3
TR-Z2	BF38
TR-Z5	BF45
TR-27	BF2
TR-28	BF20

Out of the 13 faecal samples there are only 4 samples amplified via nested PCR and annotated as successful. The overall success of all nested PCR applied samples were calculated as 57.14%.

3.2 Data Analysis

3.2.1 Genetic diversity

From a total of 35 mtDNA 281bp control region sequences, 14 distinct haplotypes were revealed (Table 3.6).

Table 3.7: Samples represented in 14 distinct haplotypes

Haplotype ID	**Sample ID**
H1	TR-1; TR-2; TR-3; TR-4; TR-5
H2	TR-Z1
H3	TR-6; TR-7; TR-8; TR-9; TR-10; TR-11; TR-12; TR-13; TR-14; TR-15; TR-16
H4	TR-17; TR-18
H5	TR-19; TR-20
H6	TR-21
H7	TR-22; TR-Z3; TR-Z4

Table 3.7 (cont'd)

Haplotype ID	Sample ID
H8	TR-23
H9	TR-24
H10	TR-Z6
H11	TR-25
H12	TR-26
H13	TR-Z2; TR-Z5
H14	TR-27; TR-28; TR-29

H1-7 fell into the previously identified clade 3 (Subclade 3a) while H9 and H10 fell into clade 1 (Subclade 1b). H8 appeared to be a member of the previously presumed extinct Lebanon clade of Calvignac *et al.* 2009. However, in this study by following/extending the nomenclature of Leonard et al. 2000, subclade 1d was assigned for the concerned haplotypes. Moreover, the clade previously named 'Iran' was renamed as clade 7 because the origin of H11-14 was Turkey and this situation proved that the clade's geographic distribution is not restricted to Iran. Also, H11 and a captive specimen from a Greek Zoo (Miller *et al.*, 2006) split from the remaining Turkish clade 7 specimens and other samples belonging to Clade 7. Therefore these two haplotypes (H11, captive one in Greek Zoo) were distinguished as subclade 7b whereas the other branch was denoted as subclade 7a (Fig 3.3).

Figure 3.3: Distribution of samples, belonging to four different maternal lineages, throughout Turkey. Dark blue, orange, light blue, green and yellow colors represent subclades 1b, 3a, 1d, 7b and 7a respectively.

Table 3.8: Summary statistics of molecular diversity indexes for particular clade or subclade in which Turkish brown bear haplotypes are present.

bp of the dataset	Group	# of Sequences	# of Haplotypes	# of Polymorphic Sites	Haplotype Diversity	Nucleotide Diversity
255	Group 7 Middle East (7a+7b)	13	8	30	0.9231 ± 0.0500	4.0730% ± 2.2414%
255	Subclade 7a Middle East (TR)	6	3	2	0.7333 ± 0.1552	0.3611% ± 0.3372%
227	Subclade 3a (Holarctic)	58	37	27	0.9528 ± 0.0182	3.2763% ± 1.7298%
281	Subclade 3a (TR)	27	7	8	0.7667 ± 0.0685	1.0012% ± 0.6131%

In addition to nucleotide and haplotype diversity calculations, pairwise group mean distances of identified groups were calculated.

Table 3.9: Pairwise group mean distances of six different groups including Turkish samples. Lower diagonal shows mean distances and upper diagonal shows standard deviations.

Groups	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**
Subclade3a (Holarctic) (1)		0.014	0.014	0.013	0.017	0.015
Subclade1b (Italo-Balkan) (2)	0.069		0.008	0.013	0.012	0.011
Subclade1a (3)	0.074	0.029		0.012	0.013	0.011
Subclade7a (Middle East) (4)	0.060	0.059	0.054		0.016	0.011
Subclade1d (Taurus-Lebanon) (5)	0.068	0.032	0.041	0.062		0.017
Subclade7b (Middle East-divergent) (6)	0.071	0.047	0.043	0.038	0.057	

3.2.2 Phylogenetic analysis

3.2.2.1 Model of DNA sequence evolution

The Hasegawa-Kishino-Yano (HKY) mutation model with gamma distributed site rate variation, using 4 discrete mutation classes, and a percentage of invariant sites (HKY+G+I) was chosen as DNA sequence evolution model by using the software jModelTest. Due to the comparison of several models and calculating AIC and BIC for all models, HKY+G+I model gave the smallest AIC and BIC (3145.9902 and 3880.9379).

3.2.2.2 Bayesian phylogenetic tree and divergence times

Phylogenetic tree constructed with 269bp mtDNA control region sequences including downloaded brown bear and out-group sequences is shown below.

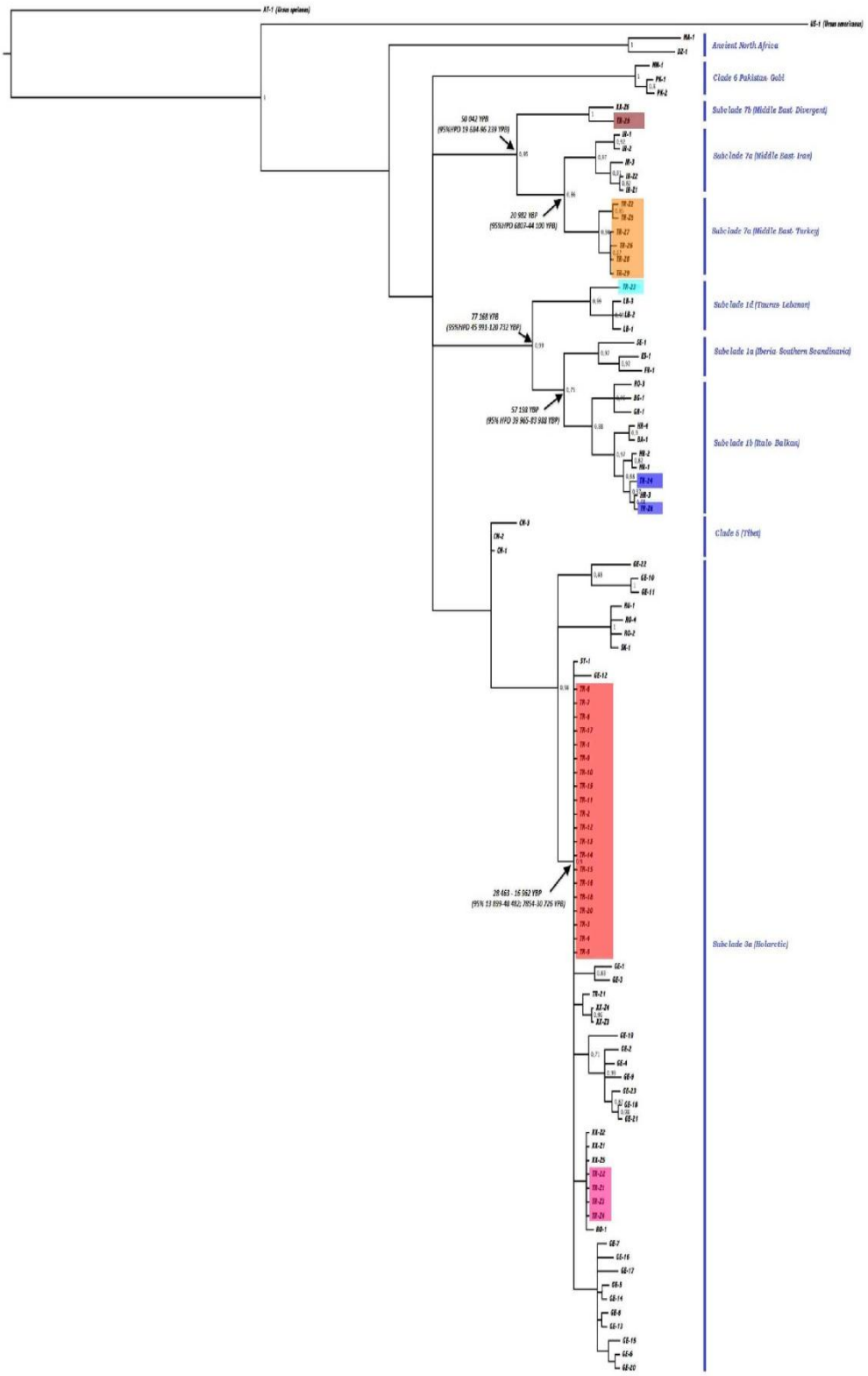

Figure 3.4: Bayesian phylogenetic tree. The Turkish haplotypes are coloured. The colours are coded in accordance with the scheme used in Figure 3.5.

As seen in Fig 3.3, TR-15 and XX-Z6 (the captive specimen from Greek Zoo) jointly split off from 5 Iranian specimens (7a Middle East-Iran) and 6 Turkish specimens (7a Middle East-Turkey) with a high posterior probability of 0.95. Moreover two geographically distinct subgroups of subclade 7a (Turkey-Iran) are separated with a posterior probability of 0.86.

TR-23 and three other Lebanon specimens (members of subclade 1d) jointly split off from subclade 1b and 1d, with a support of 0.99. Also split of subclade 1b and 1a is supported with 0.75 posterior probability.

Lastly, although the split of subclade 3a from other extant specimens could not be stated clearly in terms of time to the most recent ancestor (TMRCA), posterior probability of this split was calculated as 0.9.

On the other hand, TMRCA calculations of the highlighted split nodes above are summarized in Table 3.10.

Table 3.10: TMRCA findings of highlighted nodes on Fig.3.3

Groups	**TMRCA (95% HPD YBP)**
Subclade 7a- Subclade 7b	50,042 YBP (19684-96239)
Subclade 7a_Iran- Subclade 7a_Turkey	20,982 YBP (6807-44100)
Subclade 1d- Subclade 1a + 1b	77,168 YBP (45991-120732)
Subclade 1a- Subclade 1b	57,198 YBP (39965-83988)
Subclade 3a- Extant	16,962- 28,463 YBP (7854-30726 / 13899-48482)

3.2.2.3 Median joining network

The median joining network of appropriate mtDNA haplotypes can be seen below.

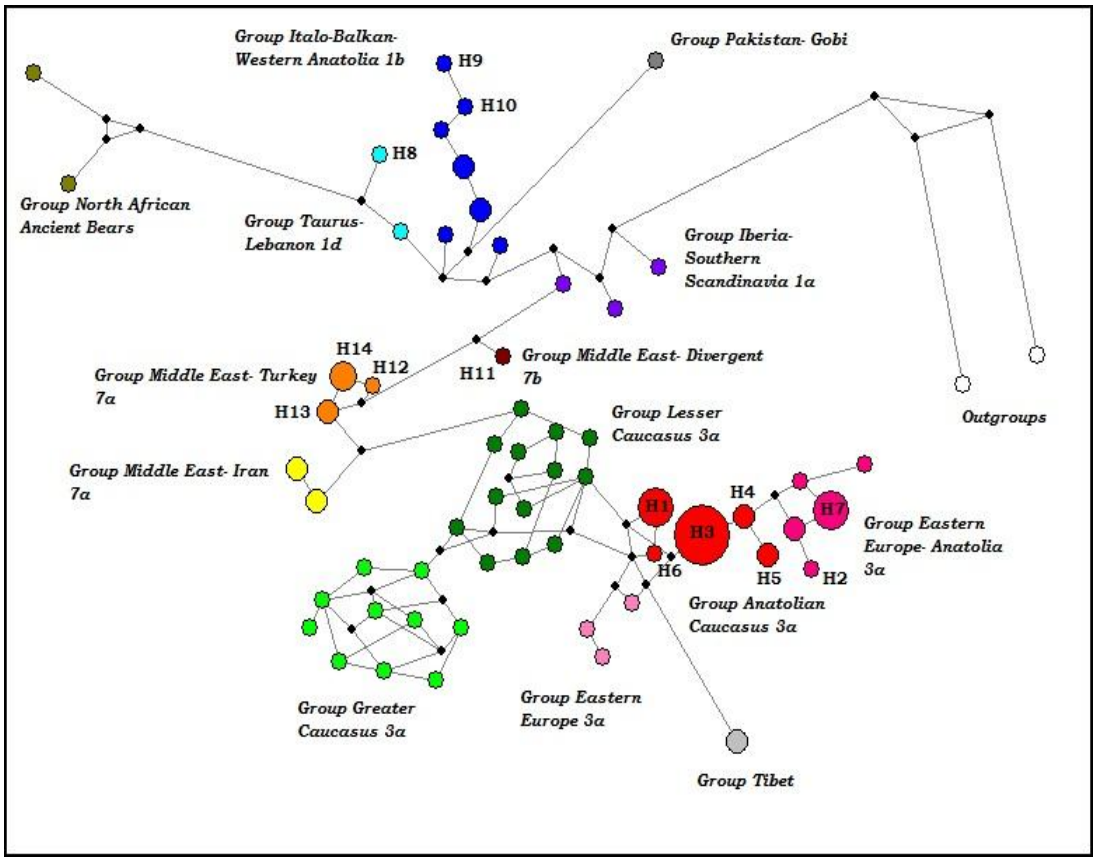

Figure 3.5: The median joining network of appropriate mtDNA haplotypes. Turkish haplotypes are annotated. Different colours represent groups treated similarly in Fig. 3.4

The median joining network supported the outcomes from the Bayesian phylogenetic tree. For example, Turkish haplotypes within clade 3a were connected to Eastern European specimens and to Caucasus (Lesser and Greater) specimens. Subclade 7a (Middle East-Iran) and subclade 7a (Middle East-Turkey) groups are closely connected to each other; however, subclade 7b (Middle East-divergent) seems to be much more distant. Also sister subclades 1a, 1b and 1d form a group together in contrast with their distance to clade 7 and subclade 3a.

CHAPTER 4

DISCUSSION

4.1 DNA Isolation and PCR

Guanidinium thiocyanate (GuSCN) is a chaotropic agent which is known by its strong protein denaturant role (Boom *et al.*, 1990). Since faecal DNA samples are potentially carry inhibitors because of lipid, carbohydrate etc. occurrence in faeces of the animals, GuSCN associated DNA isolation procedures are frequently used in non-invasive genetic sampling studies (Taberlet & Luikart, 1999).

In this study, there were five distinct types of faecal DNA isolation procedures applied to the samples. The procedures that obtained the highest DNA concentrations were GuSCN + Qiagen DNeasy Blood & Tissue Kit and GuSCN + Qiagen QIAamp Stool Kit, probably due to the protein denaturant role of GuSCN leading to the disruption of other cells (such as plants or other animals consumed by the target species). Since DNA concentration was evaluated by its optical density, not only the target DNA but also total DNA concentration in the sample was measured (Beja-Pereira *et al.*, 2009). Therefore, the high amount of DNA obtained from faecal samples reflects this condition, and should be kept in mind when PCR success rates of various methods are compared. For example although the average concentration of NORGEN Stool DNA Isolation Kit is higher than that obtained using Qiagen QIAamp Stool Kit, PCR success rates for both methods' are calculated to be equal (20%). A similar case is that of Zymo Research Fecal DNA Mini Prep versus GuSCN + Qiagen QIAamp Stool Kit: Although the former gave a lower concentration of DNA, PCR success percentages for both methods were equal (13.33%). The highest PCR success rate (33.33%) was obtained using a combination of GuSCN and Qiagen DNeasy Blood & Tissue Kit. When the kit procedures except Qiagen Blood & Tissue Kit's

is considered, main conclusion about their common point could be that they all contain GuSCN containing DNA wash buffers which DNeasy Blood & Tissue Kit does not include (see manufacturer instructions of the Kits mentioned). Therefore, it may be claimed that this difference might have occurred because GuSCN was not completely eliminated from the eluted DNA and interfered with the downstream process afterwards. However, it is worthy to note that the number of samples for such an analysis was low. The main conclusions from such data could be that, isolated DNA concentrations are not directly related to overall PCR success and the success rates belonging to each procedure are comparable with values from the literature. For example, Qiagen QIAamp Stool Kit was reported to give really high success rates when compared with GuSCN-silica, digest buffer/phenol-chloroform and lysis buffer/spin column menthods (Wasser *et al.*, 1997; Waits & Paetkau, 2005; Bhagavatula & Singh, 2006), but in this study, interestingly, this procedure was the second most successful one.

Faecal DNA amplification is designated as problematic in terms of low concentration of target DNA, degradation of DNA, and the presence of inhibitors in the DNA extracts (Deuter *et al.*, 1995; Kohn *et al.*, 1995). In order to comment on degraded DNA, or in other words, in order to eliminate this problem, the state of the faecal material is highly important (De Barba *et al.*, 2010). During fieldwork, it may be hard to evaluate the freshness of faeces. There are studies showing that even the length of the field survey may have an influence on the amplification process (Skrbinšek *et al.*, n.d). Therefore well planned field surveys seem to be crucial. On the other hand, there are suggested solutions for the inhibition problem, summarized in Beja-Pereira *et al.* (2009). They are dilution of the DNA extracts prior to amplification, including with Bovine Serum Albumin (BSA), Tween 20 or Triton- X 100 into PCR tubes, and precipitation of DNA with EtOH. In this study, 1:300 to 1:1000 dilutions of the samples were applied; however, no improvement in amplification success was observed. Precipitation of DNA with EtOH prior to elution step was also applied in order to eliminate inhibitors and concentrate DNA prior to PCR. However, this method was not successful either. In addition, although BSA was often suggested for the elimination of inhibitors, BSA (0.1%) included PCR reactions all denoted as failed due to the shining well views on the photographs of the agarose gels. Controlled experiments including one

reaction with positive control DNA and 0.1% BSA, and one reaction with the same DNA sample but without BSA, were performed PCR. However, the agarose gel electrophoresis results indicated that BSA including reaction's well was shining instead of a clear band as represented by positive control reaction. Eventually, BSA use was terminated due to observed and verified failure. Although unlikely, the reason for this failure could be the destabilization/denaturation of BSA in laboratory conditions in a very short time. Therefore, it is highly important to conduct further controlled experiments with BSA or other inhibitor eliminating chemicals in order to increase the amplification success for DNA obtained from faeces. Another method using starch (Zhang *et al.*, 2006) also seems worthy to try and compare with the methods mentioned above.

The necessity of pre-amplification of low-quality samples is equivocal. Although there are some studies suggesting pre-amplification (such as Piggott *et al.*, 2004; Bellemain & Taberlet, 2004), there are others indicating that it may be unnecessary depending on the condition of the samples (De Barba & Waits, 2010; Skrbinšek *et al.*, 2010). In this study nested PCR was applied for low-quality faecal DNA samples and there were 4 low-quality DNA samples from which DNA was managed to be amplified and reliable sequences obtained (overall success of nested PCR was calculated as 57.14%). If the treated samples contained degraded DNA, nested PCR was expected not to work, because the outer primer set encompasses 400bp long, whereas the inner primer set encompasses 269bp long DNA. With degraded DNA, it should be expected that the longer primer would not work properly; however when the success rate of the nested primer (57.14%) is considered, the problem seems not to lie with degraded DNA, but probably with inhibitors interfering with the amplification reactions, highlighting the importance of the inhibitor eliminating procedures once more.

When the average concentrations of DNA obtained from non-invasively collected samples (faeces, hair and old tissue) are considered, overall concentration obtained from hair samples significantly differed from the ones obtained from other DNA sources (see Table 3.3). On the one hand, the highest calculated success rate belongs to hair samples with 34.69% (followed by faeces, 30.61%, and old tissue, 22.22%). Hair samples, by the nature of the hair bulbs, do not

contain inhibitors like faeces samples (Goossens *et al.*, 1998). Moreover, single stranded DNA, the main PCR interfering agent in old tissues, is not a concern for hair samples either (Pääbo, 1989; Pääbo *et al.*, 2004). Therefore, the only factor decreasing the PCR success rate for hair samples seems to be the extremely low amounts of extracted DNA. The number of hair bulbs would directly affect the amount of DNA (Taberlet *et al.*, 1999). In this study, Chi-square statistics for hair samples including <10 hair bulbs vs. including >20 in terms of sample succession, indicated significant difference at p=0.002 and supported the former statement. As well as the number of hair bulbs, the freshness of the bulbs should also be considered in order to increase the sample success rate of hair samples, because it is inversely correlated with degradation of DNA. 34.69% sample success of hair samples which are at least 3 week-old, could be stated as considerable regardless of the number of hair bulbs per sample, because this success rate can increase up to 80% when the hair samples are at most 2-week old (F. Davoli, pers.comm.). If the field surveys cannot be arranged properly to assume the freshness of the samples, then the appearance of the hair bulbs could be considered as a freshness determining factor. For example, if a hair bulb looks dark and dry instead of whitish and wet, this may be linked with an increased probability of degradation. One way to overcome degradation of hair samples might be to treat them as soon as they are collected. The Chelex 100 protocol (Walsh *et al.*, 1991) could be suitable for such an application, because samples could be treated easily with Chelex 100 even in the field (Woods *et al.*, 1999). However during field surveys, the storage problems should also be considered. Therefore, freshly collected hair samples are essential for PCR success regardless of the extraction procedure itself. For hair samples that failed possibly due to degraded and/or trace amount of DNA (Navidi *et al.*, 1992; Taberlet *et al.*, 1996), designing shorter base pair comprising primers is another solution. For example, with the help of multiple tubes approach these newly designed primer sets (using 3-4 primers instead of two primers for 269 bp CR) could be used with the same DNA extract's PCR replicates. Amplification of smaller sequences would theoretically work better (<200-300; Taberlet *et al.*, 1999) so that sequences derived from smaller fragments could be united during the DNA alignment step. On the other hand, there is one study dealing with hair samples in which improvement of DNA extraction was proven by the use of calcium ions for the increase of digestion

and the release of DNA (Pfeiffer *et al.*, 2004). This new technique could also be used for the improvement of DNA extraction which would directly affect the PCR success rate due to mentioned drawbacks in the use of hair samples in non-invasive genetic sampling studies.

Old tissue samples used in this study were all dried skin fragments which were treated with salts (aluminium sulphate or copper sulphate) for long term storage. Along with the general problem of possibly degraded single strand DNA in the extracts (Pääbo, 1989; Höss *et al.*, 1996; Hofreiter *et al.*, 2001 etc.) it was also important to eliminate these salts (Matheson *et al.,* 2009). For the elimination, high concentration of EDTA (10M) was used; however, EDTA itself could be a major problem in downstream processes (Kreader, 1996). The lowest sample success rate (22.22%) among non-invasively collected samples belongs to old tissue samples. There are only two successful old tissue samples that yielded reliable sequences for phylogenetic analysis and all those sequences were extracted with NORGEN Stool DNA Isolation Kit. It can be speculated that the success might be due to the ability of this Kit's spin columns in eliminating single stranded DNA as well as due to the strong lysis steps associated with GuSCN (see manufacturer's instructions). The lysis step is important because even if the metal ions coming from the salts in the skin fragments were assumed to be eliminated by EDTA, dried skin is hard to lyse and GuSCN associated two-step lysis of this Kit would allow increased levels of cell disruption. Also, it seems that eventually a high concentration of EDTA can be eliminated from samples through the DNA washing step of NORGEN Stool DNA Isolation Kit. Other solutions that might improve success in ancient DNA amplification include the dilution of DNA extracts prior to downstream reactions (for example as applied in Gilbert & Wilson, 2004), using single strand eliminating DNA recovery filters such as GENECLEAN® For Ancient DNA Kit, Qbiogene, Inc., Carlsbad, California (see Höss & Pääbo, 1993; Scandura *et al.*, 2006), or multiple primer set use, encompassing smaller fragments.

In Table 3.4, overall sample success percentages were shown as well as the % DNA extraction and % PCR success values of distinct type of DNA sources utilized in this study. When non-invasively collected samples were taken into account, decrease in % success in each consecutive step could be observed. In

other words, for faeces, hair and old tissue samples the largest success rate was obtained from DNA extraction, followed by PCR and the overall sample success rate. Mean reason behind this situation could be the low amount of target DNA in hair and/or old tissue samples, high amount of bulk DNA in faeces samples and the low quality DNA dilemma for all these sample types.

Non-invasive genetic sampling requires caution at each step because small amounts of DNA are prone to several types of errors during amplification (Taberlet *et al.*, 1999). In order to track possible errors in DNA extraction and PCR processes, positive and negative controls were used. For DNA sequencing, forward and reverse strands were sequenced and aligned jointly for verification. Moreover, if a unique haplotype was detected, its DNA extraction was replicated at least twice, and the samples were sent for sequencing at least twice again for the verification. Haplotypes that were obtained more than once were verified by their multiple occurrences and by other checks.

The only invasively collected sample type used in this study was fresh tissue, in the form of a small piece of skin obtained from bears under anesthesia. Although DNA was extracted from each sample (n=6), the overall success rate was 83%, considerably lower than the predicted value of 100% success. The main reason for the observed failure could be a mistake in the lysis step. Most probably, the lysis step applied to that sample failed and cell disruption was completed properly ending up with the failure in PCR.

All in all, for non-invasively collected samples, there are further possible PCR and/or reliable sequencing enhancing procedures that may lead to improved results. However, in order to make scientifically meaningful suggestions or calculations of efficiency, systematically designed field surveys are needed in addition to the need of considerably increased number of samples.

4.2 Genetic Diversity and Phylogenetics

In this study 35 mtDNA CR sequences were obtained including 14 distinct haplotypes. In order to control if the same haplotypes were whether from same individual or not, samples' geographic proximity were compared. In addition,

information about microsatellite analysis (comprising the genotyping of 11 loci) of some common samples was checked.

There was a single haplotype previously reported (Talbot & Shields, 1996) from northeastern Turkey. This reported haplotype belonged to Subclade 3a while haplotypes revealed in this study did not only belong to Subclade 3a, but also to Clade 1 and Clade 7, therefore revealing a high level of maternal lineage diversity in Turkey. In addition, when the haplotype diversity of Group 7 (7a + 7b), Subclade 7a (Middle East-Turkey), Subclade 3a (Holarctic) and Subclade 3a (Turkey) were considered (Table 3.8), a high level of genetic diversity within Turkish brown bears was revealed, despite the limited number of available samples.

Bayesian inference approach was preferred for the representation of phylogenetic relationships between genetically diversified samples obtained from Turkey and other samples from Holarctic. This preference was depending on the much more structured pattern observed in Bayesian phylogenetic tree when it was compared with the ones constructed with Neighbor-Joining and Maximum Likelihood methods. Although, locations of clades were mainly concordant between these trees, structures of the subclades could not be supported by high posterior probabilities and there were some misinterpretations between previously identified subclade specimens (see Appendix A for detailed comparison of the structures).

Specimen TR-23 (H8, Table 3.6), collected from Akseki, Antalya, clustered with an ancient sample originating in Lebanon reported in Calvignac *et al.* (2009) (Fig. 3.3) and they together formed a divergent subclade named 1d. Valdiosera *et al.* (2007) reported 3 distinct haplotypes from France which could be defined as transient haplotypes within Subclades 1a and 1b. Although there was no nomenclatural suggestions for them in Valdiosera *et al.* (2007), Davison *et al.* (2011) mentioned these transient haplotypes as belonging to Subclade 1c. Genetic mean distance between Subclade 1a and 1b was calculated as 2.9% ± 0.8% whereas it was 4.1% ± 1.3% between Subclade 1d and 1a, and 3.2% ± 1.2% between Subclade 1d and 1b. The higher genetic distances to either of the sister subclades prove that the Lebanon samples from Calvignac *et al.* (2009) and TR-23 could be classified jointly as a distinct subclade and named as 1d.

Specimen TR-23 is located in the Taurus Mountains that extend on an east-west axis along southern Turkey and is linked to the coastal mountains in Syria, Lebanon via the Amanos chain. This geographical connectivity and the occurrence of a specimen belonging to Subclade 1d indicate that in the past Taurus- Levant populations were probably connected.

There were two other haplotypes, TR-24 (H9; Table 3.6) from southern (Akseki, Antalya) and TR-Z6 (H12) from northwestern (Uludağ, Bursa) Turkey, both of which are closely related to bears from the West Balkans, especially to those from Croatia (Taberlet & Bouvet, 1994; Kocijan *et al.* 2011); hence these haplotypes clustered within Subclade 1b. Although the eastwardmost record of Clade 1 (Western Europe) was previously reported from Greece (Taberlet & Bouvet, 1994; Korsten *et al.* 2009), these findings indicated that the range of Clade 1 extends further eastwards. Also, occurrence of subclades 1b and 1d was reported near Akseki on the Taurus Mountains, showing the only known case of the sympatry of these two subclades.

There are four distinct haplotypes (H11-14) belonging to 7 specimens originating in Turkey, which clustered within Clade 7 (Figure 3.3). Most of these specimens with known origins were collected from northeastern Turkey, so far restricted to an area bounded in the north by River Çoruh. The two Clade 7 specimens collected from zoos (TR-Z2 and TR-Z5) showed slightly different haplotypes. Since their origins were not certain, Clade 7 individuals may not be restricted only the extreme northeastern Turkey. In Figure 3.3 it is clearly seen that samples originating from Iran and those from Turkey form distinct branches. The mean genetic distance of these two groups is calculated as 1.7% ± 0.8%. This value is low compared to distances between subclades (for instance, between subclade 1a and 1b, seen above). Therefore, these two groups were designated as two different populations within Subclade 7a. On the other hand, H11 revealed from specimen TR-25 (BH3; Table 3.5) clustered with a captive specimen from a Greek zoo (Miller *et al.*, 2006) and jointly formed a novel lineage named Subclade 7b. The exact origin of the captive specimen is not known, but the person who collected this sample did so because this individual “looked unusual” (L. Waits, pers.comm.). It is unlikely to have an origin in Greece because so far the only clade found in Greece was Clade 1 (Taberlet & Bouvet, 1994; Korsten *et al.*, 2009) while the origin of the most

similar TR-25 is Artvin in the northeast of Turkey. The mean genetic distance among subclades 7a and 7b was calculated as 3.8% ± 1.1%, providing evidence for the distinction of these groups as different/divergent subclades.

Seven haplotypes were found to belong to Subclade 3a within the Turkey. Subclade 3a samples occur throughout the current bear range in Turkey, except for the Taurus Mountains (see Fig. 1.2 and Fig. 3.4). Although Calvignac *et al.* (2009) reported a Syrian ancient sample from a geographically nearby locality, in this study no haplotypes belonging to Subclade 3a was reported from the Taurus Mountains. The available data may indicate historical or on-going current presence of Clade 3, but fail to reject its absence. Strong geographical structure within Clade 3a is demonstrated in Eastern Europe and Siberia by Taberlet & Bouvet (1994), Kohn *et al.* (1995), Korsten *et al.* (2009) as well as in Caucasus, based on a dense sampling by Murtskhvaladze *et al.* (2010). However, in Turkey no such strong geographical structure was observed (Fig. 3.4). The most interesting result dealing with specimens belonging to Subclade 3a was that most of the specimens belonging to Greater and Lesser Caucasus populations reported by Murtskhvaladze *et al.* (2010) cluster separately than any bear specimens found in Turkey (see Fig. 3.5). Actually, only one specimen (GE-12) clusters with Turkish 3a specimens. However, this situation might be explained by the strong female philopatry seen in brown bears (Randi *et al.*, 1994; Waits *et al.*, 1998; Støen *et al.*, 2005). On the other hand, a cluster with RO-1 haplotype (Taberlet & Bouvet; 1994) and samples from western Turkey was observed, indicating a connection between Anatolian and Balkan populations.

Contact zones of brown bear clades were previously reported in East Carpathians (Clade 1 and 3, Kohn *et al.*, 1995; Zachos *et al.*, 2008), and in Scandinavia (Subclades 1a and 1b, Taberlet *et al.* 1995). In Anatolia, in addition to the overlap between subclades 1b and 1d in southwest, Subclade 3a overlaps with Subclade 1b in the northwest and with Subclade 7a in the northeast. Although these overlapping structures or general phylogeographic pattern of brown bears in Turkey are not totally concordant with any other species', overlap of eastern and western clades in southwestern part of Anatolia was also observed with lesser white-toothed shrew (see Fig. 4 at Bilgin, 2011).

Therefore, overlapping distributions of brown bear clades is not uncommon in Turkey. In contrast with the evidence from recent phylogeographic pattern observed in Europe (Davison *et al.*, 2011), this may reflect various lineages coexisting for a long time with little impact due to cycles of population contraction and expansion during the last ice age. It has been proposed that some late Pleistocene samples belonging to the lineages of ancestral clades 1 and 3 were observed in Europe more than 100,000 years BP (Hofreiter *et al.*, 2004; Valdiosera *et al.*, 2008). Also, both clades represented overlapping phylogeographic structure both in Central Europe and Iberia. Therefore, this sympatric occurrence of some divergent clades supported the conclusion that the degree of phylogeographic structure currently observed in Europe did not exist prior to the LGM (Davison *et al.*, 2011).

During the LGM, tundra and permafrost covered most of the places in Central Europe and Iberian and Italo-Balkan peninsulas had suitable habitat and temperate species providing role. Therefore recently allopatric subclades 1a and 1b were believed to be isolated during the conditions of LGM mentioned before (Taberlet *et al*, 1998). However, recent findings showed that despite the benign conditions, in Central Europe or even northern parts of Europe occurrence of brown bears and some other species (see the list in Davison *et al.*, 2011) were verified. Moreover, gene flow between refuge samples was also shown by some researches (Valdiosera *et al.,* 2007; 2008). So that, once more it was highlighted that clade overlaps observed few thousand years ago in Europe might not be represented by today's phylogeographical structure observed in the same area.

TMRCA estimations of clades and subclades would provide opportunities for linking the particular climatic periods and the evolution of brown bears. However, in this study, the confidence intervals of TMRCA estimates were too wide to safely comment on splits, especially about clusters including smaller number of samples (such as subclades 7a and 1d). Still, estimates calculated in this study can be compared with previously published results, and turn out to be compatible with them. For instance, the split of Subclade 1d (Taurus-Lebanon) from the Western European group (1a and 1b) was found to occur about 77.000 years BP (95% HPD: 45,991-120,732 YBP) which is congruent with the suggestion of Calvignac *et al.* (2009) as c. 65.000 years BP. The estimated time between Subclade 1a and Subclade 1b was about 57,198 YBP

(95% HPD: 39,965-83,988 YBP) which falls among the time ranges suggested by Calvignac *et al.* (2009), Davison *et al.* (2011) and Ho *et al.* (2008). Also, the time between subclade 7b (Middle East-divergent) and 7a (Middle East) was calculated to be 50,042 YBP (95% HPD: 19,684-96,239 YBP).

In contrast to these older divergence times reported above, the estimated time for the split between the two population groups of Subclade 7a (namely, Middle East-Turkey and Middle East-Iran) was found to be 20,982 YBP (95% HPD: 6,807-44,100) whereas the time split within subclade 3a (Holarctic) excluding two groups (Lesser Caucasus and Eastern Europe) was suggested to be 28,463 – 16,962 YBP (95% HPD: 13,899-48 482; 7,854-30,726 YBP) respectively. Lower limit of the estimated interval is concordant with the Last Glacial Maximum (LGM) and with the findings of Murtskhvaladze *et al.* (2010) which is about 20,000 YBP.

Climatic events that occurred in the past are expected to have a deep influence on the amount and distribution of intraspecific variation in brown bears (Taberlet *et al.*, 1998). Moreover, the geographical proximity of animals and the genetic similarities of their mitochondrial DNA sequences are expected to be correlated (Hofreiter *et al.*, 2004). Based on those perspectives, previously it was claimed that colonization routes of brown bears may represent an extraction/contraction model, representing the pattern of recolonization from peninsular refugia (Taberlet & Bouvet, 1994; Taberlet *et al.*, 1998; Hewitt, 2000). However ancient brown bear DNA research has revealed that populations presumed to be isolated (in refugia) showed gene flow in between; thus, rather than the result of a simple model, brown bears may today display a complex phylogeographic structure (Hofreiter *et al.* 2004; Valdiosera *et al.*, 2007) with which historical and current phylogeographic pattern of the species could be explained with the help of some modifications and/or additions such as considering series of bottlenecks in northern populations resulted with decreased genetic diversity (Hewitt 1996; 2000) and/or considering the stochastic processes occurred because of exponentially increasing human activity in Holocene (Valdiosera *et al.*, 2007; 2008).

All in all, in this study, 14 newly identified haplotypes belonging to clades 1, 3 and 7 prove the importance of Turkey as a source of bear genetic diversity and

imply a key role for Turkey in the possible post-glaciation colonization of brown bears. However, it is obvious that additional sampling from eastern and western Turkey is crucial for a better understanding of the phylogeography of this species, and to obtain statistically significant results in mean genetic distance calculations and TMRCA estimations. Increased sampling not only from Turkey, but also from Iran, Iraq and Transcaucasia is required for a better assessment, particularly of Clade 7 and Clade 3.

CHAPTER 5

CONCLUSIONS

Non-invasive genetic sampling is an approach which allows genetic studies dealing with wild animals without catching or even observing them. Therefore it decreases the efforts on field surveys conducted for elusive animals such as brown bears. In Turkey brown bear is one of the largest mammals on which genetic studies are lacking significantly. So that with the success rates of several distinct DNA isolation procedures and PCRs calculated in this study play a significant role on the improvement of non-invasive genetic sampling studies in the future.

In this study 35 brown bear mtDNA control region sequences and 14 distinct haplotypes were identified. These haplotypes were belonging to three different clades named as Clade 1, Clade 3 and Clade 7 encompassing five distinct subclades, 1a, 1b, 3a, 7a and 7b which indicates the high genetic diversity in brown bear maternal lineages found in Turkey. Moreover, previously clade/subclade distribution borders were reconstructed because the only brown bear subclade occurrence had been reported as subclade 3a. In addition to that additional clade overlaps were reported with subclade 1d & 1b and subclade 3a & 7b. On the other hand, although intervals of TMRCA calculations are wide for the construction of phylogeographic scenarios of some clade splits, main clade split times were concordant with the ones reported in the literature which points out the reliability of constructed analysis.

All in all, this study reports the high genetic diversity in maternal lineages of Turkish brown bears and indicates the significance of widely distributed increased number of samples in Turkey and as well as in the neighborhood for the increased resolution in the phylogeography of the animals.

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APPENDIX A

SAMPLES OF PHYLOGENETIC TREES

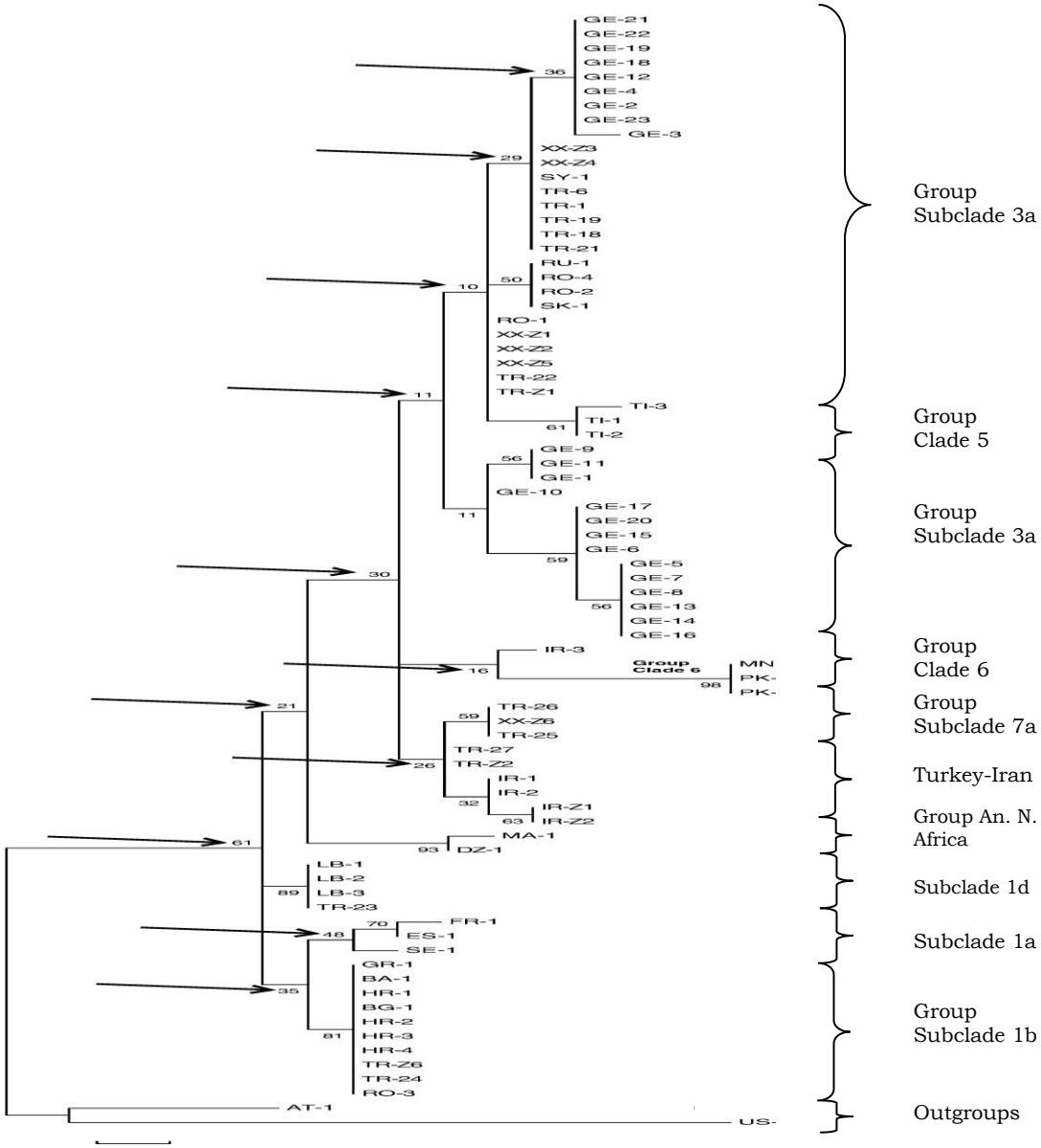

Figure A.1: A phylogenetic tree constructed with Maximum Likelihood method.

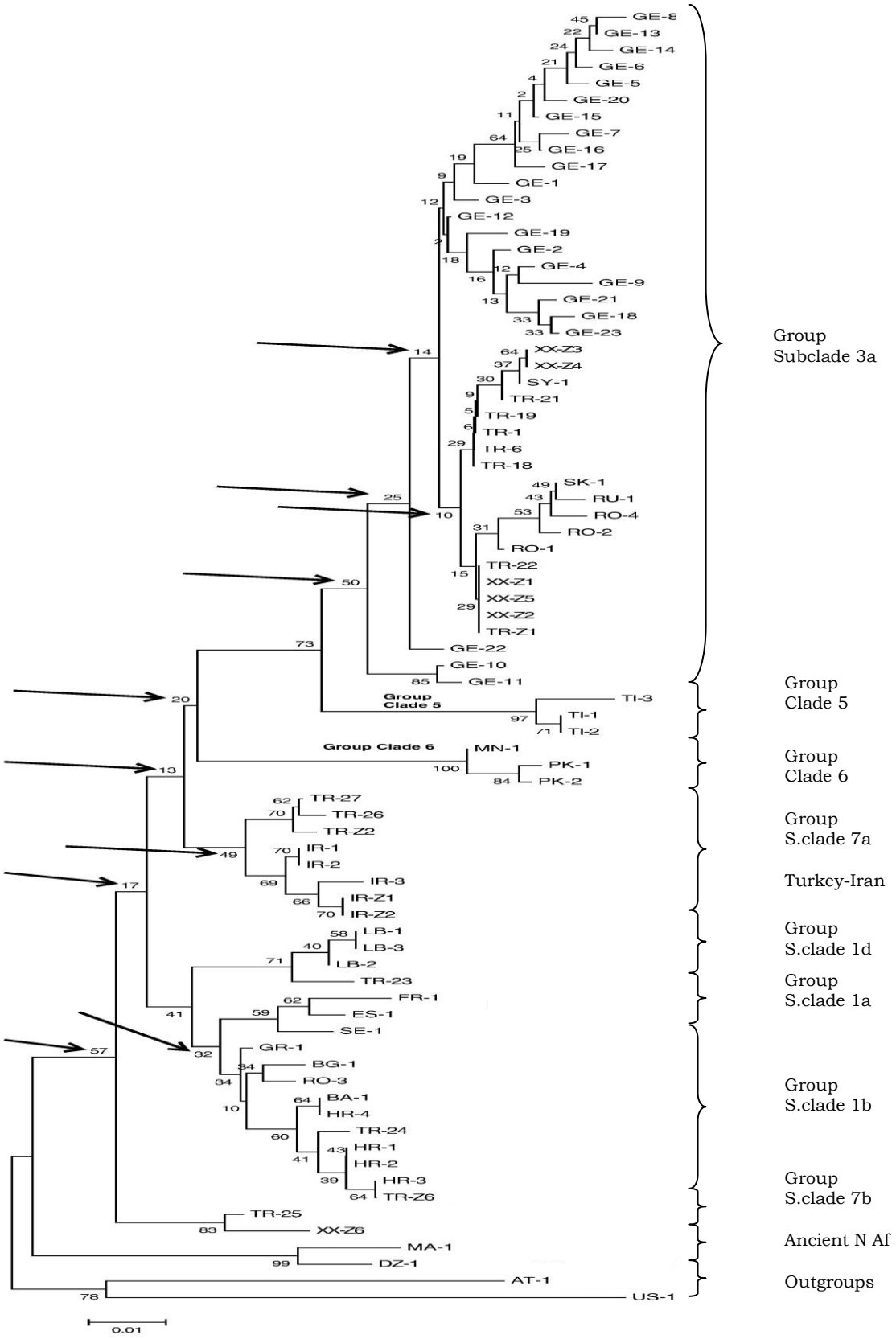

Figure A.2: A phylogenetic tree constructed with Neighbor- Joining method