MITOCHONDRIAL DNA (MTDNA) SEQUENCE ANALYSES OF KANGAL DOGS IN TURKEY

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_____

Prof. Dr. Canan ÖZGEN

Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

_____

Prof. Dr. Semra KOCABIYIK

Head of the Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

_____

Prof. Dr. İnci TOGAN

Supervisor

Examining Committee Members

Prof. Dr. Zeki KAYA	(METU, BIO)	_____
Prof. Dr. İnci TOGAN	(METU, BIO)	_____
Prof. Dr. Okan ERTUĞRUL	(Ankara Ünv., Vet. Fak.)	_____
Assoc. Prof. Dr.Vahdettin ALTUNOK	(Selçuk Ünv., Vet. Fak.)	_____
Assist. Prof. Dr. A. Elif ERSON	(METU, BIO.)	_____

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Çiğdem GÖKÇEK

ABSTRACT

MITOCHONDRIAL DNA (MTDNA) SEQUENCE ANALYSES OF KANGAL DOGS IN TURKEY

Gökçek, Çiğdem
M.Sc., Department of Biological Sciences
Supervisor: Prof. Dr. İnci Togan

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Kangal dogs are the most popular dogs of Turkey due to their strength, intelligence, loyalty, endurance to extreme temperatures and their lack of predatory behavior towards livestock.

In this study to provide genetic information about the distinctness of Kangal and Akbash dogs and hence to provide a basis to conserve them separately, 585 base pair of the mitochondrial DNA (mtDNA) control region sequence was analysed in 105 Kangal and 9 Akbash dog samples.

All the results indicated that Kangal and Akbash dogs were different from each other. Comparison of the Turkish data with those from other dogs revealed that Kangal dogs harbour a rare haplogroup which is only seen in Scandinavian (36%),

Portuguese (20%), Turkish (20%) dogs and only one Spanish dog, but not in Akbash, Middle Eastern, other European, Eastern Asian and Indian dogs. Furthermore, comparison of the Kangal and Akbash dogs with the dogs from different geographic regions indicated that Kangal dogs are genetically closer to Scandinavian and South West Asian dogs whereas Akbash dogs are more similar to European and East Asian dogs, based on the mtDNA control region sequences

Today, the sizes of Kangal and especially Akbash populations are decreasing. An urgent program for their conservation is needed. In order to conserve them separately, it must be understood that these two dogs are genetically distinct. That is why, the main purpose of the present study is to provide genetic information about the distinctness of Kangal and Akbash dogs.

Keywords: : Kangal dog, Akbash dog, canine, mtDNA, genetic distnictness

ÖZ

TÜRKİYE'DEKİ KANGAL KÖPEKLERİNİN MİTOKONDRİAL DNA (MTDNA) DİZİ ANALİZİ

Gökçek, Çiğdem
Yüksek Lisans, Biyoloji Bölümü
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Gücü, zekası, sadakati, zor hava şartlarına dayanılıklığı ve çiftlik hayvanlarına karşı yırtıcı davranışı olmayışından dolayı Kangal köpekleri Türkiye'nin en popüler köpeklerinden biridir.

Sunulan çalışmada Kangal ve Akbaş köpeklerinin genetik farlılığını ortaya çıkarmak amacıyla 585 baz uzunluğundaki mitokondriyal DNA kontrol bölgesi için 105 Kangal ve 9 Akbaş köpeği örneklerinden sekans analizi yapılmıştır

Sonuçların hepsi, Kangal ve Akbaş köpeklerinin birbirlerinden farklı olduklarına işaret etmektedir. Türkiyeden elde edilen verilerin diğer köpek verileri ile karşılaştırılması Kangal köpeklerinin sadece İskandinavya (%36), Portekiz (%20), Türkiye (%20) köpeklerinde ve sadece bir İspanya köpeğinde bulunan ama Akbaş,

Orta Doğu, Avrupa, Doğu Asya ve Hindistan köpeklerinde bulunmayan çok nadir bir haplogruba sahip olduğunu göstermiştir. Yine, Kangal ve Akbaş köpeklerinin mtDNA kontrol bölgesi sekanslarına dayanarak farklı coğrafik bölgelerdeki köpekler ile karşılaştırılması Kangal köpeklerinin genetik olarak İskandinavya ve Kuzey Batı Asya köpeklerine, buna karşılık Akbaş köpeklerinin Avrupa ve Doğu Asya köpeklerine daha yakın olduğunu göstermiştir

Günümüzde, Kangal ve özellikle Akbaş köpeklerinin sayısı azalmaktadır. Acil bir koruma programı gereklidir. Bu köpekleri ayri koruyabilmek için genetik olarak da birbirlerinden farklı oldukları kanıtlanmalıdır. Bu nedenle Kangal ve Akbaş köpeklerinin genetik olarak birbirlerinden farklı olduklarını göstermek sunulan çalışmanın temel konusunu oluşturmaktadır

Anahtar Kelimeler: Kangal köpeği, Akbaş köpeği, köpek, mtDNA, genetik farklılık

.

To My Family..

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LIST OF ABBREVIATIONS

α : Alpha

β : Beta

κ : Kappa

π : Nucleotide Diversity, Pi

θ : Theta

Φ: phi

°C : Degrees celsius

µl : microliter

µM : Micromolar

A : Adenine

AMOVA: Analysis of Molecular Variance

bp : base pair

B.P : Before Present

C : Citosine

ddATP : Dideoxyadenosine Triphosphate

ddCTP : Dideoxycytidine Triphosphate

ddGTP : Dideoxyguanosine Triphosphate

ddNTP : Dideoxynucleotide Triphosphate

ddTTP : Dideoxythymidine Triphosphate

dH_2O : Distilled water

DNA : Deoxyribonucleic acid

DnaSP : DNA Sequence Polymorphism

dNTP : Deoxyribonucleotide Triphosphate

EDTA : Ethylene diamine tetra acetic acid

EtOH : Ethanol

e.g: For example

f: Female

FCI: Fédération Cynologique Internationale

G : Guanine

Hd: Haplotype Diversity

HPG: Haplogroup

ID: Identity Card

K_3EDTA : Potassium EDTA

Kimura-2P : Kimura-Two Parameter

m: Male

M : Molar

MEGA : Molecular Evolutionary Genetics Analysis

mg : miligram

$MgCl_2$: Magnesium Chloride

ml : Mililiter

mM : Milimolar

MSN : Minimum Spanning Network

mtDNA : Mitochondrial DNA

NaAc : Sodium Acetate

ng : Nanogram

NJ : Neighbor Joining

OTU's : Operational Taxonomic Units

PCR : Polymerase Chain Reaction

pp: Page

pH : Potential of hydrogen

pmol : Picomoles

r: Raggedness Statistic

Rpm : Rotations per minute

RT : Room temperature

S: Segregating Sites

SDS : Sodium dodecyl sulfate

T : Tymine

TAE Buffer : Tris Acetate EDTA Buffer

Taq : *Thermus aquaticus*

UV : Ultra Violet

V : Volt

CHAPTER I

INTRODUCTION

In this chapter first information on domestic dog, the archaeological and molecular finds about the origin of domestic dog, the reason of using mtDNA sequences to understand the phylogenic relationship of the populations will be given; and then history of livestock guarding dog will be summarized. Finally, the aims of the study will be presented.

1.1. The Domestic Dog, *Canis familiaris*

"Wild dog crawled into the Cave and laid his head on the Woman's lap…And the Woman said, 'His name is not Wild Dog any more, but the First Friend,' Rudyard Kipling, Just So Stories" (Acland and Ostrander, 2003).

The dog, *Canis familiaris*, was the first animal domesticated by humankind (Clutton-Brock, 1995) and it is the species that has been most profoundly altered through selective breeding (Hart, 1995). The dog is beloved as humankind's most faithful companion (Acland and Ostrander, 2003). People have been closely associated with dogs for many thousands of years (Hart, 1995) and they often mention that they feel a sense of calmness and security around their pets (Katcher, 1985 referred by Hart, 1995). Humans have the tendency to personify dogs, give

human-sounding names to their dogs and treat them like family members. Although people sometimes used them as working dogs, the close relationship between man and dog has been affectionate rather than utilitarian (Morey and Wiant, 1992 referred by Coppinger, 1995). Dogs can provide significant companionship for children as well as for adults. Children commonly use their pets for comfort when they are feeling bored, lonely and unhappy (Kidd and Kidd, 1985 referred by Hart, 1995).

The domestic dog is a member of the family Canidae, a diverse group of carnivores that is divided into 38 species. For example; wolf, jackal, fox, dingo are some of the species classified in this family. The 38 species classified within the family Canidae are listed below (see Table 1.1). It is the only member of the family that can be said to be fully domesticated (Clutton-Brock, 1995). Today, there are more than 1000 breeds identified and they are widespread around the world (Morris 2001 referred by Bannash et al., 2005). Because of the fact that the domestic dog is the most variable member in terms of size, shape and behavior, it has been assumed in the past that multiple species of canids have shared in the ancestry of the domestic dog.

Table 1.1. The 38 species classified within the family Canidae are listed below (Taken from Clutton-Brock, 1995, Page 9).

Canis lupus, wolf	Europe, Asia, N. America, Arctic
Canis familiaris, dog	Worldwide
Canis familiaris dingo, dingo	Australia
Canis rufus, red wolf	Central N. America
Canis latrans, coyote	N. America
Canis aureus, golden jackal	SE Europe, N. Africa, S. Asia
Canis mesomelas, black-backed jackal	Africa south of the Sahara
Canis adustus, side-striped jackal	Africa south of the Sahara
Canis simensis, Simien jackal	Mountains of Ethiopia
Alopex lagopus, arctic fox	Arctic
Vulpes vulpes, red fox	Europe, N. Africa, Asia, N. America
Vulpes corsac, corsac fox	Central Asia
Vulpus ferrilata, Tibetan fox	Tibetan plateau
Vulpus bengalaensis, Bengal fox	India
Vulpus cana, Blanford's fox	SW Asia
Vulpus rueppelli, Rüppell's fox	N. Africa, SW Asia
Vulpus pallida, pale fox	Sahel
Vulpus chama, Cape fox	S. Africa
Vulpus velox, swift or kit fox	N. America
Fennecus zerda, fennec fox	N. Africa, Arabia
Urocyon cinereoargenteus, grey fox	N. America, northern S. America
Urocyon littoralis, island grey fox	Islands of California
Nyctereutes procyonoides, raccoon dog	E. Asia
Dusicyon australis, Falkland Is. 'wolf'	Falkland Islands, extinct since *c*. 1880
Dusicyon culpaeus, culpeo	S. America – Patagonian subregion
Dusicyon culpaeolus, Santa Elena zorro	Uruguay
Dusicyon gymnocercus, Azarra's zorro	E. Patagonian subregion
Dusicyon inca, Peruvian zorro	Mountains of Peru
Dusicyon griseus, grey zorro	SW Patagonian subregion
Dusicyon fulvipes, Chiloe zorro	Island of Chiloe
Dusicyon sechurae, Sechuran zorro	NW Peru, Ecuador
Dusicyon vetulus, hoary zorro	Brazil
Cerdocyon thous, crab-eating zorro	S: America – Brazil subregion
Atelocynus microtis, small-eared zorro	Central S. America – Brazil
Chrysocyon brachyurus, maned wolf	Southern Brazil subregion
Speothos venaticus, bush dog	S. America – Brazil subregion
Lycaon pictus, African wild dog	Africa south of the Sahara
Cuon alpinus, dhole	E. and Central Asia
Octocyon megalotis, bat-eared fox	Africa south of the Sahara

The family Canidae has the same number of chromosomes and its members are capable of interbreeding to produce fertile offsprings (Clutton-Brock, 1995). Within this family, based on morphological and behavioral characters the domestic

dog is most closely related to wolves, jackals and the coyote. This three species were considered as the ancestors of the domestic dog until recently but the combined result of many morphological, behavioral, archaeological and especially genetic studies indicate that dogs are descendants of the wolf, *Canis lupus* (Coppinger and Schneider, 1995; Vila et al., 1997; Wayne and Ostrander, 1999; Savolainen et al., 2002; Leonard et al., 2002).

1.2. Studies on The Origin of The Domestic Dog

1.2.1. Archaeological Finds

The archaeological finds help scientists to trace and localize the first domestication sites. The earliest sites, where the remains of the wolf/dog skulls indicates a decrease in skull size and auditory bullae and crowding of teeth compared to wolf, are considered as the sites where early domestication took place (Tchernov and Horwich, 1991 referred by Clutton-Brock, 1995; Coppinger and Schneider, 1995). It has been indicated that the inevitable effect of taming is reduction in the size of body and head as it can be seen in other domesticated mammals (Clutton-Brock, 1995).

The earliest find of a domesticated dog consists of a mandible from a late Paleolithic grave at Oberkassel in Germany (Nobis, 1979 referred by Clutton-Brock, 1995) and dated back to about 14 000 years before the present (B.P). Chronologically, other archaeological finds and dates are: a puppy skeleton that had been buried with a human found at Ein Mallaha, Israel (see Figure 1.1) dated back to 12 000 years B.P; skull and neck vertebrae found at Mesolithic Sites Star Carr and Seamer Carr in England, respectively dated to 9 500 years B.P; skulls, teeth and skeletal bones found at a Neolithic site of Jarmo in Iraq and dated back to 9 250-7 750 years B.P; and some other finds unearthed at Eastern Anatolia and around Lake

Baykal in Russia from 9 000 years ago; South America from 8 500-6 500 years ago; Tacibara, Japan from 8 000 years ago (Clutton-Brock, 1995; Serpell, 1995) and a morphological feature of the jaw diagnostic of domestic dogs which is also found some Chinese wolves bu generally not in other wolves suggesting East Asian origin dating back to 7 500 years B.P (Savolainen et al., 2002).

Figure 1.1. The burial of a human with a puppy from the Natufian site of Ein Mallaha, Israel (Taken from Clutton-Brock, 1995, Page 11).

1.2.2. Genetic Studies

The dispersed archaeological finds from all over the world suggested that domestication has started more or less simultaneously at different regions of the world and different subspecies of wolf have contributed to the dog ancestry (Clutton-Brock, 1995). But the genetic studies tells a different story.

First comprehensive mtDNA study done by Vila et al. (1997) supports the archaeological records in that wolf is the ancestor of the domestic dog and several wolf subspecies have contributed to the different dog lineages. Unlike archeological record, it was estimated that the earliest dogs may have been domesticated more than 100 000 years B.P. This age is far older than any other previous estimate of the dog's age. It is also disagrees with a date recovered from a larger data set of dog and wolf mtDNA examined by Savolainen et al. (2002).

Savolainen et al. (2002) analyzed the genetic variation in 582 base pairs (bp) of mtDNA obtained from 38 Eurasian wolves, and of 654 domestic dogs from Europe, Asia, Africa, and Arctic America. All dogs were grouped in to 6 lineages (A to F) with respect to their maternal inheritance. In a recent study, done by van Asch et al. (2005) a total of 143 dogs were analysed and four major haplogroups A, B, C and D were observed. Among the clades considerably higher frequency were observed in Clade A (~70%) then B, C and D respectively. Furthermore, when the dogs from all over the world are considered the frequencies of clades A, B and C were similar in all regions which suggest that the major present dog populations had a common origin from a single gene pool containing clades A, B and C (Savolainen et al., 2002). Moreover, among the three main clades there was no clear division of the main morphologic types of dogs e.g. spitz, mastiff, greyhound which suggests that the extreme morphologic variation among dog breeds is not the result of geographically distinct domestications of wolf. Whereas in cattle, goat, sheep, lama the mtDNA studies are suggesting two, possibly three domestication events for

example, in sheep at least three possible domestications were recorded (Townsed, 2000 and Bruford and Townsed, 2004 referred by Koban, 2004).

In recent studies mentioned above, clades D, E and F were found only regionally in Turkey, Scandinavia, Portuguese and Spain; Japan and Korea; and Japan and Siberia, respectively (Savolainen et al., 2002; van Asch et al., 2005). Considering the frequeny of clade D in both studies the results are as follows: 36% in Scandinavian samples, 20% in Turkish samples, 20% in Portuguese samples and just one sample from Spain. In both studies within 24 Turkish samples there were 10 Kangal, 11 Akbash, 2 Anatolian Shepherd Dog and 1 Feral dog samples. A total of 91% of Akbash dogs had haplotypes belonging to Clade A; 9% had types belonging to Clade B; there were no haplotypes belonging to Clade C and Clade D whereas a total of 30% of Kangal dogs had haplotypes belonging to Clade A; 10% had types belonging to Clade B; 40% had types belonging to Clade C and 20% had types belonging to Clade D. At this stage of the introduction it must be emphasized that the distribution of D lineage; i.e. its presence only in Scandinavian, Turkish (only in Kangal dogs), Porteguese and Spanish samples and its total absence in Akbash dog samples is a remarkable point.

In Savolainen et al. (2002) study, due to the larger genetic variation found in East Asia, it was suggested that dogs originated about 15 000 or 40 000 years B.P from a common gene pool in East Asia, and have subsequently spread all over the world. Moreover, a study done by Leonard et al. (2002) indicated that there was no separate domestication event in the New World, both ancient and modern dogs throughout the world are descendants of the old world wolves.

The origin of the Australian dingo is another important question of interest ever since the arrival of Europeans to Australia in the 18th century (Macintosh, 1975 referred by Savolainen et. al., 2004). In order to obtain a more comprehensive picture of the origin and history of the dingo, mtDNA sequences in 211 dingoes were

examined by Savolainen et al. (2004) and compared with 676 dog samples from a world-wide. The results indicated that dingoes have an origin from domesticated dogs coming from East Asia.

1.3. Mitochondrial DNA (mtDNA) Sequences in the Studies of Populations

Using a variety of molecular tools, including maternally (mtDNA), paternally (Y chromosome) and bi-parentally (autosomal and X-chromosome microsatellites) inherited markers, it is possible to track the population's origin, to follow the changes in genetic diversity over time and to assess the impact of rare immigrants on this natural population (Vila, 2003).

Sequencing is the most informative technique, and with the growing popularity of automated DNA sequencers it is becoming much easier to implement. However, the approach has found its greatest value for phylogenetic and phylogeographic analyses, where it is necessary to have ordered characters (Lowe et al., 2004).

One of the most commonly used method for determining the evolutionary relationship between species or the history of populations within a species is the sequence analysis of mitochondrial DNA (mtDNA) (Castro et al., 1998 referred by Savolainen, 1999). The reason for this is that mtDNA as a whole has several advantages in comparison to nuclear genome such that, it has a high rate of sequence evolution, almost 20 times greater than that of nuclear DNA for synonymous sites (Pesole et al., 1999 referred by Savolainen, 1999). The high rate of sequence evolution resulted in a large degree of variation between species and between individuals (Savolainen, 1999) so that high mutation rates allow researchers to distinguish between the recently diverged lineages. Secondly, mitochondrial genome has an inefficient repair mechanism compared with that of nuclear genome. Furthermore, mtDNA does not recombine, that is the difference between any two

mitochondrial sequences represents only the mutations that have taken place since each sequence was derived from a common ancestor (Jorde et al., 1998). Transmission of mtDNA without recombination through maternal lineages enables the tracing maternal phylogenies. Thus, these special features of mtDNA make it a good tool for population genetics and the sequencing of the control region which is the most variable region in mammals can provide better resolution than other regions (Savolainen, 1999; Stoneking, 2003). The major disadvantage concerning the use of mtDNA analysis in population genetic studies is that the mtDNA is maternally inherited. This implies that studies of mtDNA reveal the history of the female population only. Analysis of the Y-chromosome (reflected by paternal lineages) has far been under-utilized to understand the evolutionary and historical relationships in animals. The study of the paternal lineage of dogs should compliment mitochondrial analysis. Based on the lack of recombination and the smaller number of males used in domestic animal breeding programs, it is predicted that Y-chromosome analysis would provide a powerful tool to study breed origins and relationships (Bannash et al., 2005). For example in Bannash et al. (2005)'s study, based on the Y chrosome network analysis it was indicated that African breeds have the most ancient haplotypes based on their deeply distance from the center which is similar to the network of human Y chromosome haplotypes (Forster et al., 2000 referred by Bannash et al., 2005).

In the present study, mtDNA control region was chosen for studying Turkish livestock guarding dog samples: Kangal and Akbash, to prove their distinctness as well as providing further information for the history of domestic dogs.

1.4. Anatolian Livestock Guarding Dogs

The early archaeological records show that the domestic dog was present in Anatolia (Asia Minor) as early as 7 000 B.C. (Nelson, 1996). These very ancient dogs' remains were found at Neolithic sites such as Hacilar in western Turkey and

Çatal Höyük on the Konya Plain of central Turkey (Walters, 1969 referred by Nelson, 1996). These remains are believed to constitute the native dog of Anatolia.

The later archaeological records show that the presence of large guarding dogs, probably of the mastiff family. There are numerous wall reliefs of Mastiff type dogs of Anatolia or as they're more commonly known, The Assyrian and Babylonian Dogs in museums around the world (Reed, 1996). The same overall appearance such as, heavy in head and chest, strong in limbs, usually with the tail curved over the back, swift and agile and wearing spiked iron collar which save the dog's throat from the teeth of the wolf, can be seen from those wall reliefs (Reed, 1996). This picture is familiar to today's Kangal dog. They are also very large and heavy dogs with a big head and chest, and their tails are curved over the back and wearing spiked iron collar.

Although, some people believe that the Kangal dog may have been descendant of those ancient mastiff dogs of Middle East, some people disagree. According to Karadağ (2002), the theory which states that the Kangal dog originated in the times of the Assyrians and the Babylonians is not true, since there are no dogs resembling this breed in those regions today.

Turkey is a land bridge between Europe in the West and Asia in the East and in the past numerous civilizations and animals passed across this ancient land (Nelson, 1996). This complex relationship among humans and animals as well as the geographic topology of the land produced the diversity of native dogs which we see in Turkey today (Nelson, 1996). Nelson (1996) has described these native breeds of Anatolia as "regional dog breeds" which is a breed of dog known to originate in, or be identified with, a specific region within a larger context-usually a country or political state. He describes the Kangal Dog is a regional breed associated with the Sivas-Kangal Region of East Central Turkey. According to Karadağ (2002), Kangal

dog is not just specific to Sivas region, it was brought into Anatolia along with the sheep flock from Turkestan during the migration of Nomadic Turks.

The general features of the Turkish livestock guarding breeds studied in the present study are as follows:

Kangal Dog: A regional livestock guarding dog breed. It is a natural dog breed associated with Sivas-Kangal region of Sivas province in central Turkey. It invariably has a black mask on a massive head and short, dense hair ranging in color from dune (yellow) to steel grey (Nelson, 1996). It has a powerful body. Its tail is slight curl when it relaxes but when it alerts its tail is curled over back. It is the most popular dog of Turkey. It is often used as guard dogs to watch over livestock, factories and houses. It is known throughout Turkey and abroad for its strength, intelligence, loyalty, endurance to extreme temperatures, and its absence of predatory behavior toward livestock (Altunok et al., 2005).

Akbash Dog: A regional livestock guarding dog breed characterized by its white color. It is a natural dog breed found in western Turkey primarily in Eskişehir and neighboring provinces. It is a livestock guarding dog used to guard sheep and other animals against predators (Nelson, 1996). It has been gentrified in the U.S.A. and in Europe almost 25 years.

Unfortunately, there are no formal written records or pedigrees for these dogs in Turkey. The recent study based on 100 canine microsatellites indicated that Kangal and Akbash dog were significantly different from each other by F_{ST} measure (Altunok et al., 2005). It should be emphasized that altough Kangal and Akbash dogs are recognized as seperate breeds by Turkey and several western kennel clubs, the Federation Cynologique International (FCI), groups Kangal and Akbash dogs, along with other Turkish guardian types, into one breed known as the Anatolian Shephard Dog with the breed number 331.

1.5. The Objectives of the Study

The sizes of Kangal and especially Akbash populations are decreasing due to decreased agricultural activities, decline in sheep industry and increasing migration of villagers to the cities (Altunok et al., 2005). An urgent program for their conservation is needed. In order to conserve them separately (as opposed to FCI) it must be proven that these two dogs are genetically distinct. Thus, the main purpose of the study is to provide genetic information about the distinctness of Kangal and Akbash dogs.

Specifically, the aims of the study are as follows:

- To sequence mtDNA control region of Turkish dog samples.
- To study the incidence of D haplogroup in a larger Kangal and Akbash populations.
- To examine the types of the D haplogroup.
- To find out the geographic distribution of the Kangal and Akbash's possessing the D haplogroup.
- To suggest a conservation program for Kangal and Akbash dogs.
- To contribute to the studies on evolutionary history of dogs.

CHAPTER II

MATERIALS AND METHODS

In this study, 114 dog samples were analysed. This chapter includes the information about the samples i.e., sampling places and sampling methods; methods employed for the laboratory and the statistical analyses. DNA extraction and statistical analysis parts of the present study were carried out in department of Biology at Middle East Technical University in Ankara, Turkey whereas sequencing part of the present study was carried out in Royal Institute of Technology (KTH) in Stockholm, Sweden.

2.1. Sampling

2.1.1. Collection Sites of Individuals

In this study 105 Kangal and 9 Akbash dog samples were analysed. Kangal samples were obtained from the villages of Sivas and Kayseri provinces. The number of Akbash samples were low because there were only 9 samples available during sampling. Out of 9 Akbash samples 3 of them were from Karacabey breeding farm in Bursa, 4 of them were from Eskisehir provinces, 1 of them was from Istanbul and 1 of them was from Aksaray. During sampling , special care was taken not the sample

from kin individuals. The details about the samples (if known), such as; sample id, name of the dog, sex of the dog, parents of the dog and the address, are provided in Appendix B.

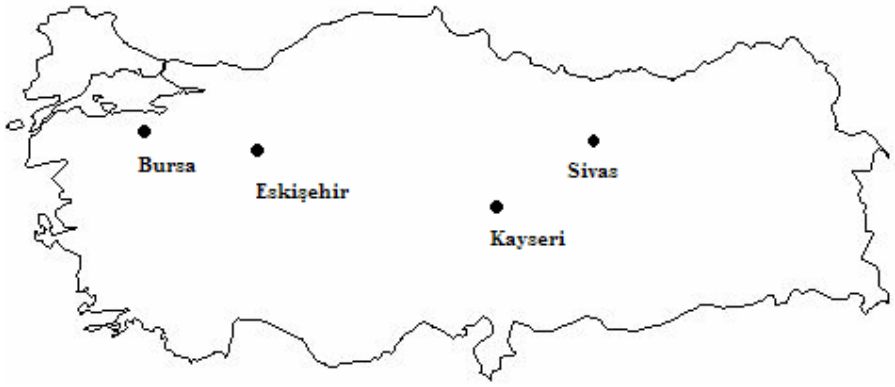


Figure 2.1. Locations of the sample sites.

2.1.2. Types of Tissue Samples

In this study, two types of samples were used: blood and buccal swap samples. Buccal swap samples, which are epithelial tissue samples from inside the cheek, were taken from each individual with the help of a brush. Epithelial cells were collected from the inside of both of the cheeks by scraping with a sterile brush for at least one minute. The scraping should be strong enough to collect as many cells as possible. The blood samples were collected in 10 ml vacuum blood tubes containing K_3EDTA for anti coagulation. In total 63 DNAs extracted from blood samples and 51 DNAs extracted from buccal swab samples were analysed.

2.2. Laboratory Analyses of the Samples

2.2.1. DNA Extraction

DNA isolation both from whole blood and buccal swap samples was performed using phenol:chloroform extraction method. For blood samples Sambrook et al. (1989)'s method was used, whereas for the buccal swap samples method provided by Dinç (2003) was used.

2.2.1.a. DNA Extraction from blood samples

Ten ml blood was put in properly labelled 50 ml falcon tubes containing 0.5 ml of 0.5 M EDTA (pH 7.5) and completed to 50 ml with 2X lysis buffer to lyse the red blood cells. Tubes were mixed for 10 minutes by inversion and then centrifuged at 3000 rpm for 10 minutes at +4°C to precipitate the nuclei. The supernatant was removed and the pellet was resuspended in 3 ml of salt-EDTA buffer by vortexing. Then, 0.3 ml of 10 % SDS and 150 µl proteinase-K (10 mg/ml) were added and the tubes were incubated at 55°C for 3 hours. After the incubation, 3 ml phenol was added, the tube contents were mixed first vigorously for about 20 seconds, then gently for about 2 minutes and then centrifuged at 3000 rpm for 10 minutes at +4°C. The supernatant was taken into new, sterile and properly labelled falcon tubes mixed with 3 ml phenol:chloroform:isoamylalcohol solution (25:24:1). Again, the tube contents were mixed first vigorously for about 20 seconds, then gently for about 2 minutes and then centrifuged at 3000 rpm for 10 minutes at +4°C. Afterwards, the upper phase was collected carefully and transferred into a new, sterile and properly labelled glass tube by a transfer pipette. Then, about 2 volumes of 99 % ethanol was added to precipitate and collect the DNA. The glass tube was mixed abruptly and then the DNA was transferred to 1.5 ml sterile and properly labelled eppendorf tubes containing 500 ml Tris-EDTA (TE) buffer (pH :7.5) using a glass hook and stored at -20°C until use.

2.2.1.b. DNA Extraction from buccal samples

The head of the brush containing epithelial cells was placed into a 1.5 ml eppendorf tube containing 700 µl 2X lysis buffer. The cells were digested overnight at 37°C by adding 10 µl proteinase-K (20 mg/ml). After digestion, the brush was not removed since it still contained DNA. The solution was extracted first by adding 500 µl phenol:chloroform:isoamylalcohol solution (25:24:1). After mixing well it was centrifuged at 10000 rpm for 2 minutes at room temperature (RT) and the upper aqueous phase was transferred into a new tube. Then, 500 µl chloroform:isoamylalcohol (24:1) was added to the tube, mixed well and centrifuged at 10000 rpm for 2 minutes at RT. The upper phase was collected into a new tube and 0.1 volume of 3 M Sodium Acetate (NaAc) together with 0.6 volume of isopropanol were added to the solution. The tube was incubated overnight at -20°C. Afterwards the DNA was precipitated by centrifuging at 10000 rpm for 10 minutes. The pellet was rinsed with 500 µl 70% ethanol and the DNA was precipitated again by centrifuging at 10000 rpm for 5 minutes at RT. After discarding the supernatant, the pellet was dried at RT and resuspended in 100 µl of Tris-EDTA (TE) buffer. The DNA samples were stored at -20 °C.

2.2.1.c. Checking the DNA on the Gel

After DNA extraction, the DNAs quality was checked on agarose gel electrophoresis. An agarose gel (1 %) was prepared by melting agarose in 1X TAE buffer. Three µl of isolated genomic DNA, 4 µl of 6X loading buffer and 17 µl of dH_2O were mixed and then 8 µl of this mixture was loaded into the wells. The gel was run at 150 V for about 20 minutes. It was then visualized under UV light. The presence of DNA bands (intact DNA), smears (if any broken DNA) as well as the thickness of the bands (quantity) were checked. The DNA bands were compared with a DNA band of known concentration to decide about the concentrations of the samples, which is then used to adjust the concentrations of the sample DNAs prior to

amplification. Furthermore, this information is used to decide how many µl of DNA sample should be put into the PCR mixture.

2.2.2. Amplification of Mitochondrial DNA (mtDNA) Control Region by Nested Polymerase Chain Reaction (Nested-PCR)

For this study, 585 bp of the dog mitochondrial genome were analysed. Amplifications of the mtDNA control region were carried out using nested-PCR. In this method the larger fragment produced by the first round of PCR is used as the template for the second PCR. The sensitivity and specifity of DNA amplification can be dramatically increased by using this method. This technique almost eliminates any nonspecific amplification products. This is because after the first round of PCR if the wrong locus were amplified by mistake, it is unlikely that it would also be amplified a second time by a second pair of primers (Newton and Graham, 1994).

An initial reaction with forward primer H15404 (5′-cct aag act caa gga aga agc) and reverse primer L16102 (5′-aac tat atg tcc tga aac cat tg), was followed by a second reaction with inner primers H15430 (5′-tcc acc atc agc acc caa ag) and L16092 (5′-ctg aaa cca ttg act gaa tag c). The names denote the 3′ end positions of the primers, according to the revised sequence submitted in 2000 by Kim et al. to the GenBank, with accession number U96639 and GI number 7534303. The initial PCR mixture consisted of 30-50 ng DNA extract, 10 pmol of each primer, 2 mM $MgCl_2$, 200 µM dNTP, 1X PCR buffer (invitrogen), and 1 unit Platinum Taq DNA polymerase in a total volume of 50 µl. The reactions were performed in ThermoHybaid MBS 02S (Thermo Electron Corporation) PCR machines. The outer PCR program consisted of a predenaturation step of 2 minute at 94°C, 25 cycles of denaturation (94°C, 30 second), primer annealing (59°C, 30 second) and extension (72°C, 1 minute), followed by a final extension at 72°C for 10 minutes. The inner PCR mixture was as follows: 1 µl of outer PCR product, 5 pmol of each primer, 2

mM $MgCl_2$, 200 µM dNTP, 1X PCR buffer (Invitrogen), and 1 unit Platinum Taq DNA polymerase in a total volume of 46 µl. The inner PCR program was identical to the outer PCR program with the exception that 30 cycles were performed. After the inner PCR, the PCR products were checked on the 1 % agarose gel and photographed using Biorad Gel Imaging System. The positive strong inner PCR products were further used for sequencing reactions. The outer and inner PCRs of the samples, which did not yield any PCR product at all or resulted in a weak product, were repeated.

2.2.3. Sequencing PCR, Cleaning of the Sequencing PCR Products and Data Collection of Sequences

Sanger's (1974) chain termination method was employed for sequencing the mtDNA control region of the samples using two primers were used for each strand. The Sanger method is based on the 5'-to-3' elongation of DNA molecules by DNA polymerase. It generates a series of single-stranded DNA molecules of differing lengths. It uses a series of four reactions, each in a separate tube, to determine the sequence of the nucleotides in a DNA segment (Klug and Cummings,1997).

In sequencing PCR, the two inner PCR primers; H15430 and L16092 were used as well as the additional two primers; H15706 (5'-cac cat gcc tcg aga aac cat) and L15791 (5'-atg gcc ctg aag taa gaa cc). Thus, there were 4 sequencing reactions for each of the sample. For the cycle sequencing reaction; 1 µl of the inner PCR product was mixed with 3 µl of 1X Cycle Sequencing buffer (Applied Biosystems), 1µl of Big Dye (Big Dye Terminator Cycle Sequencing Ready Reaction kit v3.1, Applied Biosystems) and 0.5 µl of primer in a total volume of 20 µl. The reactions were run on ThermoHybaid MBS 0.2S (Thermo Electron Corporation) PCR machines. The cycle sequencing program consisted of 30 cycles of denaturation (96°C, 10 second), primer annealing (55°C, 15 second) and extension (60°C, 4 minute). After the cycle sequencing reaction was complete, the PCR products were

purified by two steps of ethanol precipitation. They were added 50 µl of 95 % ethanol and 2 µl 3 M sodium acetate pH 5.2 for precipitation and incubated at RT for 30 minutes. After the incubation, the samples were centrifuged at 3200 rpm at 12°C for 30 minutes. The supernatant was discarded carefully. Then the products were washed with 75 µl of 70 % ethanol. After centrifuging the samples for few seconds at 700 rpm at 12°C, the ethanol is discarded and the DNA samples were dissolved in 20 µl sterile distilled water. Furthermore, the samples were first vortexed, then centrifuged for few seconds at 700 rpm for spin down before loaded on to the ABI3700 Automated DNA Analyzer machine (Applied Biosystems), which uses fluorescent detection system. Each ddNTP is labeled with a different fluorescent color in the Big Dye Terminator Cycle Sequencing Ready Reaction kit: ddCTP is blue, ddATP is green, ddTTP is red and ddGTP is black. Consequently, unlike silver staining or radioactive staining, which requires separate sequencing reactions for each of the ddNTPs; the sequencing reactions for each of the ddNTPs take place in a single tube in fluorescent labeling method. These fluorescent colors are detected by the laser incorporated in ABI3700. Thus, the base sequence data of each sequencing reaction run on ABI3700 was automatically stored on the computer during the run. After the run was complete, the data were transferred to the internal database. Then, the sequence data were available for further editing.

2.3. Editing the Raw Sequence Data

As indicated above, 2 pairs of primers were used for sequencing of each strand of the inner PCR product. So, there were 4 sequencing PCR products for each sample; 2 for the heavy strand and 2 for the light strand. The DNA sequences were first edited by using Sequencing Analysis v 2.1.1 software (Applied Biosystems). The low quality sequences were trimmed from both ends to ease further alignment. Then, all the sequences were imported into Sequencher 4.1 software (GeneCodes). The sequences were then assembled into contigs of 4, which are the 4 sequences of the same sample. The aligned sequences in the contigs were compared with each

other in terms of the base sequence. For the mislabellings, the electrophenograms of the sequences were compared, in which the sequences are represented as a series of peaks and each peak represents a base, and corrections were made. After the necessary corrections, a consensus sequence of the four was formed.

When consensus sequences were formed for each of the sample, they are first aligned and compared by using SeqEd (Applied Biosystems). Then they are imported into SeAl software (http://evolve.zoo.ox.ac.uk/software.html?id=seal), which is used to save the sequences in fasta format for further statistical analysis.

2.4. Statistical Analyses

2.4.1. Construction of Neighbor-Joining (NJ) Tree

The Neighbor-Joining (NJ) method is one of the simplest distance-based methods. It begins by choosing the most closely-related sequences and then adding the next most distance sequence as a third branch to the tree thus minimize the total length of tree. Unlike the unweighted pair-group method with arithmetic mean (UPGMA) which assumes equal rates of evolution for all taxa, NJ is designed to correct the unequal rates of evolution in different branches of the tree. In addition, NJ does not produce a dendrogram (ultrametric distance) but an additive tree (additive distance).

MEGA 3.1 software (http://www.megasoftware.net/mega.html) was used to construct NJ trees from the sequence data based on Kimura-two parameter model.

2.4.2. Kimura-Two Parameter (Kimura-2P) Distance

To estimate the number of nucleotide substitutions, it is necessary to use a mathematical model of nucleotide substitution. The assumption that all nucleotide substitutions occur with equal probability, as in Jukes and Cantor's model, is

unrealistic in most cases. For example, transitions (i.e., changes between A and G or between C and T) are generally more frequent than transversions especially for mitochondrial DNA. To take this fact into account, Kimura (1980) proposed a 2-parameter model. In this scheme, the rate of transitional substitution at each nucleotide site is α per unit time, whereas the rate of each type of transversional substitution is β per unit time. The transition : transversion ratio α / β is often represented by the letter kappa (κ). In the Kimura-2P model the number of nucleotide substitutions per site is given by ;

$$d = 1/2\ln [1/(1-2P-Q)] + 1/4\ln [1/(1-2Q)]$$

where P and Q are proportional between the two sequences due to transitions and transversions, respectively (Page and Holmes, 1998).

2.4.3. Construction of Minimum Spanning Network (MSN)

The Minimum Spanning Network is computed from the matrix of pairwise distances calculated for all pairs of haplotypes used. This network allows extant sequences as internal nodes, and the frequency with which each haplotypes occurs is indicated by the area of the circle representing it. The overall length of a network can often be shortened by adding hypothetical ancestral, unobserved nodes to the network (Jobling et al., 2004).

The Arlequin software Version 2.000 (http://anthropologie.unige.ch/arlequin) was used to compute a Minimum Spanning Network (MSN) between OTU's (Operational Taxonomic Units). By following a simple procedure below (Jobling et al., 2004), the Minimum Spanning Network can also be constructed by hand;

- a character-based distance matrix between all taxa is computed,
- links are drawn between all taxa separated by single mutational steps,
- mutational steps of increasing size are considered until all taxa are linked into a single network and all most parsimonious links of equal length from any given taxa have been reconstructed.

2.4.4. Construction of Mismatch Distribution Graphs

Mismatch distributions are graphs showing the frequency distribution of the observed number of differences between pairs of haplotypes. This distribution is usually multimodal in samples drawn from populations whose size has been constant over a long period, but it is usually unimodal in populations having passed through a recent demographic expansion (Slatkin and Hudson, 1991; Rogers and Harpending, 1992). Figure 2.2. below explains drawing a mismatch distribution graph step by step (Rogers and Harpenning, 1992).

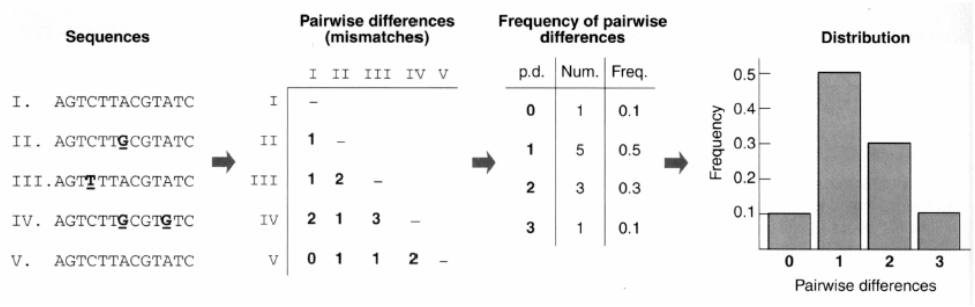


Figure 2.2. Drawing a mismatch distribution graph.

The DnaSP 4.0 software (http://www.ub.es/dnasp) was used to construct mismatch distribution graphs under the infinite site model. The model is based on three parameters: Theta Initial (θ_0, theta before the population growth or decline), Theta Final (θ_1, theta after the population growth or decline), and τ (Tau) is the date of the growth or decline measured in units of mutational time (Rogers and Harpending, 1992). It is assumed that t generations ago, a population at equilibrium

of size N_0 entered a demographic expansion phase to instantaneously reach a new size N_1 and it remained at that size ever since (see figure 2.3) and it is formulated as follow:

$$F_i^{\infty}(\theta_1,\theta_0,\tau) = F_i(\theta_1) + \exp(-\tau\frac{(\theta_1+1)}{\theta_1})\times\sum_{j=0}^{i}\frac{\tau^j}{j^!}\left[F_{i-j}(\theta_0) - F_{i-j}(\theta_1)\right]$$

where $\theta_0=2N_0u$, $\theta_1=2N_1u$, $\tau=$ 2ut, N is the population size, t is the time in generations, and u is the total mutation rate per sequence and per generation.

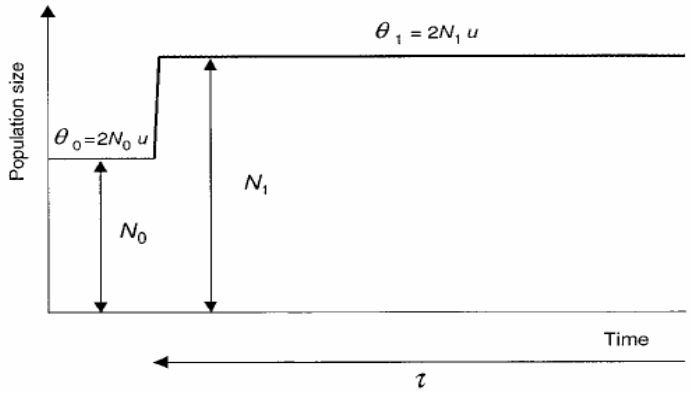


Figure 2.3. Diagrammatic representation of the instantaneous demographic expansion model (taken from Schneider and Excoffier, 1999).

DnaSP shows the distribution of the observed pairwise nucleotide site differences and the expected values in a stable population i.e. population with constant population size and it also shows the distribution of the observed pairwise nucleotide site differences and the expected values in growing and declining populations (Slatking and Hudson, 1991; Rogers and Harpending, 1992).

DnaSP estimates the raggedness statistic, r (Harpending et al., 1993 referred by Hull and Girman, 2005). This statistic quantifies the smoothness of the observed pairwise differences distribution.

$$r = \sum_{i=1}^{d+1} (x_i - x_{i-1})^2$$

where d is the maximum number of observed differences between haplotypes, and the x's are the observed relative frequencies of the mismatch classes. This index takes larger values for multimodal distributions commonly found in a stationary population than for unimodal and smoother distributions typical of expanding populations.

Nevertheless, the raggedness statistic has low statistical power for detecting population expansion. Therefore, it is better to employ a range of neutrality statistics (Ramos-Onsins and Rozas, 2002).

2.4.5. Neutrality Tests

All of the below calculations were computed using DnaSP 4.0 software (Rozas et al., 2003) to detect traces of past population growth or stability based on DNA sequences (Ramos-Onsins and Rozas, 2002).

2.4.5.a. Fu and Li's D* Test

The D* test statistic is based on the differences between η_s, the number of singletons (mutations appearing only once among the sequences), and η, the total number of mutations (Fu and Li, 1993).

2.4.5.b. Fu and Li's F* Test

: The F* test statistic is based on the differences between η_s, the number of singletons (mutations appearing only once among the sequences), and k, the average number of nucleotide differences between pairs of sequences (Fu and Li, 1993).

Fu and Li's (1993) D* and F* statistics were calculated for comparison with Fu's Fs.

2.4.5.c. Fu's Fs Test

The Fs test statistic is based on the haplotype (gene) frequency distribution conditional the value of θ. Fu's (1997) Fs test statistic uses information from the haplotype distribution to test specifically for population growth and has been shown to be among the best statistics for detecting population growth in comparisons of statistical power (Ramos-Onsins and Rozas, 2002). If Fs is significant and D* and F* are not, then population growth or range expansion is indicated, whereas the reverse suggests selection (Fu, 1997 referred by Peck and Congdon, 2004).

2.4.5.d. Tajima's D test

The D test statistic proposed by Tajima (1989) is used for testing the hypothesis that all mutations are selectively neutral (Jobling et al., 2004). The D test is based on the differences between the number of segregating sites (S) and the average number of nucleotide differences (π). Population expansion can cause significant negative departures of Tajima's D from 0 (Tajima, 1989).

2.4.6. Measuring Nucleotide Diversity (π)

For DNA sequence data, a more appropriate measure of polymorphism in a population is nucleotide diversity, π. It is the average number of nucleotide differences per site between any two randomly chosen sequences (Graur and Li, 2000). It describes the probability that two copies of the same nucleotide drawn at random from a set of sequences will be different from one another (Jobling et al., 2004).

The Arlequin software Version 2.000 (http://anthropologie.unige.ch/arlequin) was used to compute mean number of differences between all pairs of haplotypes. It is given in the following equation (Tajima, 1983),

$$\pi = \sum_{i=1}^{k} \sum_{j<i} p_i p_j d_{ij}$$

where d_{ij} is an estimate of the number of mutations having occurred since the divergence of haplotypes i and j, k is the number of haplotypes, and p_i is the frequency of haplotype i.

2.4.7. Population Pairwise Genetic Distances: F_{ST}, D_A

Two commonly used measures of genetic distance between populations are F_{ST} and Nei's standard genetic distance, D_A (Nei and Li, 1979). Both of these get values between 0 and 1, for identical and for populations that share no alleles, respectively.

The pairwise F_{ST} values are given in the form of a matrix. The P value of the test is the proportion of permutations leading to a F_{ST} value larger or equal to the observed one. The P values are also given in matrix form.

Reynold's distance (Reynolds et al., 1983) are computed from the F_{ST} values. Since F_{ST} between pairs of stationary populations of size N having diverged t generations ago varies approximately as $F_{ST} = 1-(1-1/N)^t \approx 1-e^{-t/N}$.

The genetic distance $D = -\log (1-F_{ST})$ is thus approximately proportional to t/N for short divergence times.

Nei's raw net (D_A) number of nucleotide differences between populations (Nei and Li, 1979) computed between populations 1 and 2 as

$$D_A = \pi_{12} - \frac{\pi_1 + \pi_2}{2}$$

All of the above equations were computed using Arlequin software Version 2.000 (http://anthropologie.unige.ch/arlequin).

The pairwise D_A values are given in the form of a matrix. The resulting distance matrix was then used to construct NJ trees with the program NEIGHBOR in PHYLIP 3.5c (http://evolution.genetics.washington.edu/phylip.html) (Felsenstein, 1993).

2.4.8. Analysis of Molecular Variance (AMOVA)

Differentiation between groups was also assessed by analysis of molecular variance AMOVA (Excoffier et al., 1992).

AMOVA is a hierarchical approach analogous to analysis of variance (ANOVA) in which the correlations among genotype distances at various hierarchical levels are used as *F*-statistic analogues, designated as ϕ statistics. Thus, ϕ_{ST} is the correlation of random genotypes within a population relative to those from the whole species. The statistic ϕ_{CT} is the correlation of random genotypes within a

group of populations relative to those drawn from the entire species and measures the proportion of genetic variation among groupings of populations. Lastly, ϕ_{SC} is the correlation of random genotypes within a population relative to those within a regional grouping of populations and measures the proportion of variation among populations within a region (González et al., 1998). The significance of *F*-statistic analogues was evaluated by 1000 random permutations of sequences among populations. The groupings which maximized values ϕ_{CT} and were significantly different from random distributions of individuals were assumed to be the most probable geographical subdivisions.

All estimations were performed using Arlequin, version 2000 (Schneider et al., 2000). The AMOVA design and formulae are given bellow in Table 2.1;

SSD(T) : Total sum of squared deviations.
SSD(AG) : Sum of squared deviations Among Groups of populations.
SSD(WP) : Sum of squared deviations Within Populations.
SSD(AP\WG) : Sum of squared deviations Among Populations, Within Groups.
G : Number of groups in the structure.
P : Total number of populations.
N : Total number of individuals for genotypic data or total number of gene copies for haplotypic data.

Table 2.1. AMOVA table (taken from Arlequin package program, http://anthropologie.unige.ch/arlequin).

Source of variation	Degrees of freedom	Sum of squares (SSD)	Expected mean squares
Among groups	G-1	SSD (AG)	$n''\sigma_a^2 + n'\sigma_b^2 + \sigma_c^2$
Among populations / Within Groups	P-G	SSD (AP/WG)	$n\sigma_b^2 + \sigma_c^2$
Within populations	N-P	SSD (WP)	σ_c^2
Total	N-1	SSD (T)	σ_T^2

2.4.9. List of Statistical Softwares Used and Their Webpage Addresses

The summary list of the statistical methods used in data analysis of these study and the names of the softwares used to perform these statistical tests are given (in paranthesis) below:

- Sequencing Analysis v 2.1.1 software (Applied Biosystems)
- Sequencher 4.1 software (GeneCodes)
- SeqEd (Applied Biosystems)
- SeAl software
- MEGA 3.1 software
- Arlequin 2.000 software
- DnaSP 4.0 software
- PHYLIP 3.5c

The webpage addresses for these softwares are:

- Sequencing Analysis

Applied Biosystems, http://www.appliedbiosystems.com, August 2005.

- Sequencher

Gene Codes Corporation, www.genecodes.com, August 2005.

- SeqEd

Applied Biosystems , http://www.appliedbiosystems.com, August 2005.

- SeAl

Evolutionary Biology Group, http://evolve.zoo.ox.ac.uk/software.html?id=seal, August 2005.

- MEGA 3.1

Molecular Evolutionary Genetics Analysis, www.megasoftware.net/mega.html, August 2005.

- Arlequin 2.000

University of Geneva, http://anthropologie.unige.ch/arlequin, August 2005.

- DnaSP 4.0

DNA Sequence Polymorphism, http://www.ub.es/dnasp, August 2005.

- PHYLIP 3.5c

University of Washington, http://evolution.genetics.washington.edu/phylip.html, August 2005.

CHAPTER III

RESULTS

3.1. Results of the Laboratory Experiment

3.1.1. DNA Extraction and PCR

The DNA's from all of the blood and buccal swap samples were extracted using the procedures given in Chapter II accordingly. After DNA extraction, agarose gel electrophoresis was used in order to be sure that DNAs were extracted successfully or not. Figure 3.1 represents example of DNA check gel image visualized under UV light, samples were run on 1% agarose gel. Using these images, the presence or absence of DNA bands and quantity of DNAs were checked. Some samples didn't yield any DNA (see Figure 3.1, e.g. Ak-43, K-52, K-61 in) or resulted in a weak product (see Figure 3.1, e.g. Ak-41, Ak-42, K-47, K-50, K-55). After incubating these DNA samples at 55 Cº for 20 minutes, in case they were not dissolved properly, some amount of DNA solution from these products were diluted with TE buffer in a ratio of 1:2 and then checked again. The DNA bands were compared with a DNA band of known concentration to decide how many µl of DNA sample should be put into the PCR mixture.

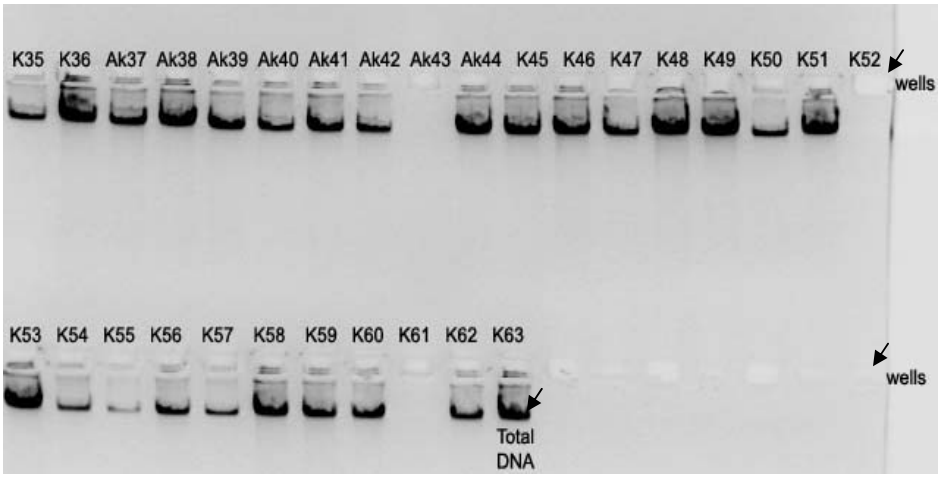


Figure 3.1. Total DNA extracts check gel.

After this procedure, amplification of sample DNAs were carried out using two-step nested-PCR amplification. The PCR products were checked on agarose gel electrophoresis and photographed under UV light. These images were used to make decisions about which samples should be sequenced and which should be reamplified as the quantity of the template is important in sequencing PCR reaction. Repeated samples which did not yield any PCR product at all or resulted in a weak product were excluded from the analysis and the strong inner PCR products (see Figure 3.2) were further used for sequencing reactions.

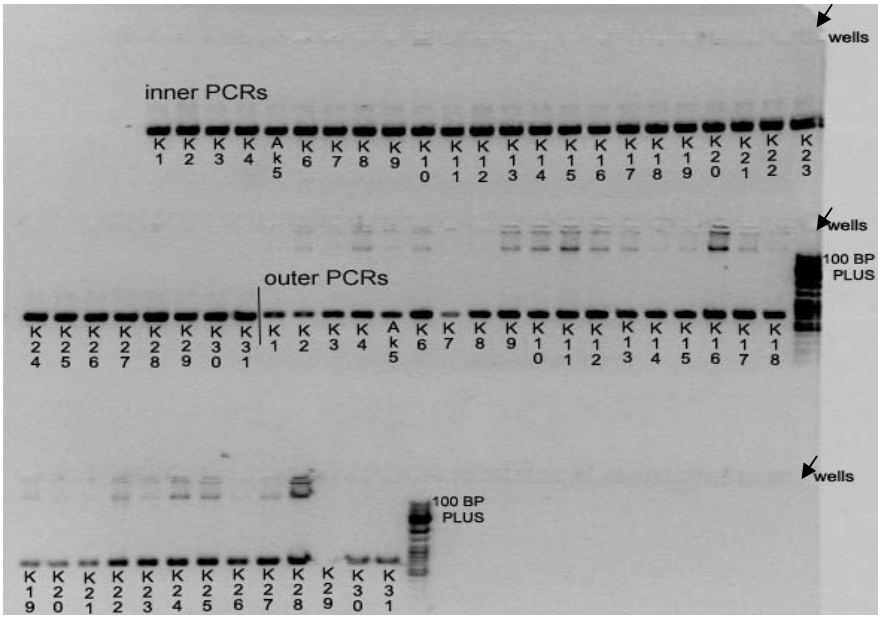


Figure 3.2. DNA amplified by nested PCR. Inner and outer PCR products of the samples in the last lane in the second raw there is a size marker which is 100 bp plus (ABI Fermentase).

3.1.2. Sequencing

For sequencing the mtDNA control region of the samples, Sanger's (1974) chain termination method was employed. Two pairs of primers were used for sequencing of each strand of the inner PCR product. Thus, there were four sequencing reactions for each of the sample; two for the heavy strand and two for the light strand. After cycle sequencing procedure, the sequencing PCR products were precipitated using EtOH and NaAc and the samples were loaded on to the ABI3700 Automated DNA Analyzer machine (Applied Biosystems), which uses fluorescent detection system.

During the run on ABI3700, the base sequence data of each sequencing reaction was automatically stored on the computer. After the run, the data were transferred to the internal database. Then, the sequence data were available for further editing. Figure 3.3 shows how the base sequence of the raw data is seen using the software Sequence Analysis (Applied Biosystems). Each ddNTP is labeled with a different fluorescent color; ddCTP is blue, ddATP is green, ddTTP is red and ddGTP is black; thus each peak representing the bases of the sequence of interest is seen in a different color.

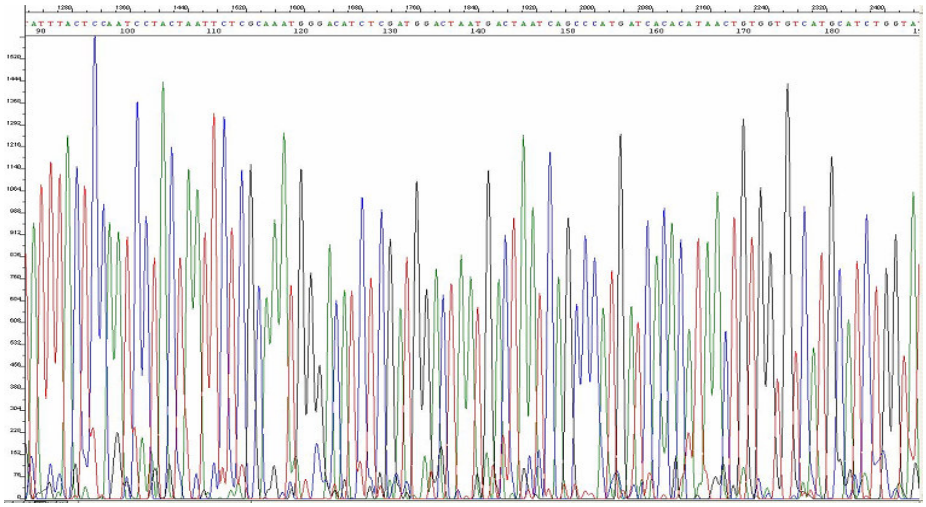


Figure 3.3: An example of the printout of an actual sequence obtained from automated DNA analyzer machine.

3.1.3. Data Editing

The resulting DNA sequences were first edited using Sequencing Analysis (Applied Biosystems). The bad sequences were trimmed from both ends of the four sequences of each sample to ease further alignment. Then, all the sequences were imported into Sequencher 4.1 software (Gene Codes), by which the sequences were assembled into contigs of four. The aligned sequences in the contigs were compared with each other for any mislabelling, as they all belong to the same individual. For example, see below Figure 3.4 (www.genecodes.com) in which the sequences were represented as a series of peaks and each peak represents a base. There are three sequences from the same individual and there is a mislabelling where the bases are highlighted. The first (reverse) and the third (forward) sequences are nice and clean sequences where the base under question is G. In the second sequence the sequencing reaction was a bit mixed up and there is C instead of G. As this sequence is not as clean as the first and third, and as the first and third are in good fit with each other, we correct the base in the second sequence as G. When comparison of the electrophenograms did not solve the mislabelling, the sequencing reactions were repeated for that sample.

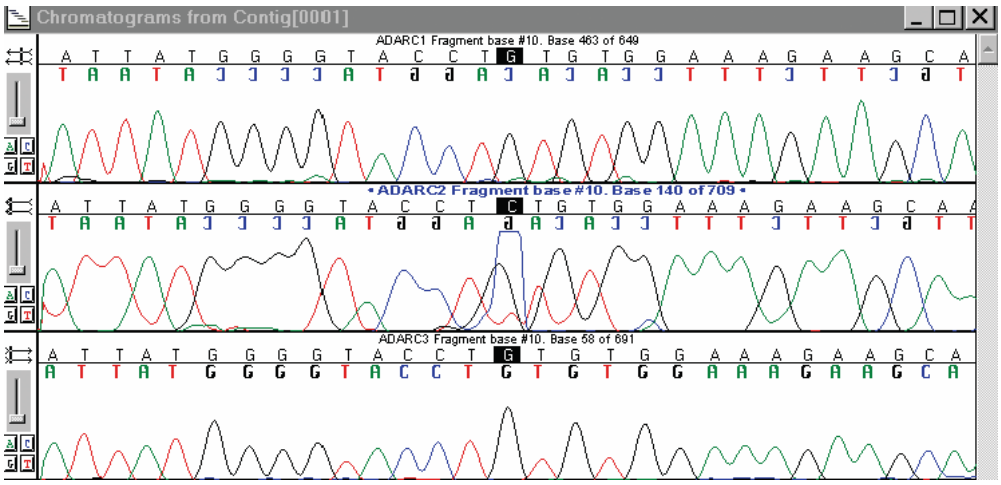


Figure 3.4. An example of the electrophenograms of the sequences.

After editing and doing necessary corrections, a consensus sequence of the four was formed for each sample. Afterwards the consensus sequences were aligned and compared using SeqEd (Applied Biosystems). Finally, they were imported into SeAl software (http://evolve.zoo.ox.ac.uk/software.html?id=seal) to save the sequences in fasta format to prepare Arlequin input file to be used in further statistical analysis.

3.2. Results of Statistical Analyses

3.2.1. Basic Statistics of the Data

The mtDNA control region sequences of the samples from Turkish Kangal and Akbash dog breeds were grouped into four clades, based on their D-loop haplotype in accordance with Savolainen et al. (2002). The sequences are compared to mtDNA reference haplotype A2 (Savolainen et al., 2002). The sites in each haplotype, which are different from this reference sequence, are shown in Figure 3.5.

In this figure, the haplotype names are given in the first column. The sequence positions of the variable sites are given above the letters representing the bases of the reference sequence. The nucleotide positions are numbered according to Kim et al. (1998). Identity with the reference sequence (A2) is denoted by a dot , substitution by a different base is denoted by the letter representing the base of interest, and each deletion is denoted by a dash.

```
                111111111111111111111111111111111111111
                555555555555555555555555555555555555666
                445555555566666666666666688888999999000
                680122222911223334455568011151345602 4
                649234568845380592635686367815418268 2
A2              TTCCTTCTCCTTTTGCTAATGATATCTTGCAGCCATT
A5              .C.............................T....
A11             .C............A.....................C.
A16             .C..........C........................
A18             .C............A..T...................
A20             .C............A..T..................C.
A22             .C.............T...................C.
A24             .C...............G.................C.
A27             .C...........CA..T...................
A28             .C............A..T...G..............
A40             .C............A.......C............C.
A49             .C..................A.............C.
A3to5step       .C..-----.............................C.
A9to3step       .C.T.....................G.....T.......
A11to1step      .C............A...........C........C.
B1              .C......TT.C..AT.GG.A...C..C.T..T.G..
B2              .C......TT.C..AT.GG.A...C..C.T.-T.G..
B10             .C......T..C..AT.GG.A...C..C.T..T.G..
B13             .C......TT.C..AT.GG.A...C..C.T..T....
B1to1step       .C......TT.C..AT.GG.....C..C.T..T.G..
B2to1step       .C......TT.C..AT.GG.A...CT.C.T.-T.G..
B3to1step       .C......TT.C..AT.G..A...C..C.T..T.G..
C2              .CT.....T.C...A..G.C....C....T.-T.G..
C5              .CT.....T.C...A....C....C....T.-T.G..
C6              -CT.....T.C...A....C....C....T.-T.G..
D5              .C............CATC.......C..CAT-.TTG.C
```

Figure 3.5. Sequence alignments of the observed haplotypes showing base substitutions and indels (-) in the mtDNA D-loop (585 bp) of Turkish Kangal and Akbash dog samples.

The observed haplotypes belonged to 4 clades, namely A, B, C and D (savolainen et al., 2002). A total of 26 different haplotypes was found in 114 dogs (n_{Kangal}=105, n_{Akbash}=9) sampled from Turkey. Six of these haplotypes were newly described. These haplotypes belong to the two major groups; namely A and B. Haplotypes A and B were found in both of the studied dogs, however, C and D were found only in Kangal dogs as indicated in Table 3.1 and 3.2. A total of 35.2 % of Kangal dogs had haplotypes belonging to Clade A, 47.6 % had types belonging to Clade B, 4.7% belonging to Clade C and 12.3 % belonging to Clade D. For Akbash dogs this proportions were as follows; a total of 88.8% of Akbash dogs had haplotypes belonging to Clade A and 11.1% had types belonging to Clade B (Table 3.2).

In table 3.1 haplogroups and haplotype distributions of the sampled dogs were indicated. In this table shaded haplotypes represent haplotypes which only observed in the samples of the present study. For the sample ID “K” represents Kangal dog and “Ak” represents Akbash dog samples.

Table 3.1. Haplogroup and haplotype distributions of the sampled dogs. For the explanation of the symbols see the text, pp 38.

CLADE	HAPLOTYPE	SAMPLE ID
A	A2	Ak-43
	A5	K-004, K-006
	A11	K-15,K-22,K-34,K-36,K-49,K-069,K-052
	A16	K-10,K-033,K-041
	A18	K-1,Ak-5,Ak-37,Ak-39,Ak-44,K-026
	A20	K-037,K-045
	A22	K-7,K-31,K-028,K-068,K-002
	A24	K-039
	A27	K-26,K-053
	A28	Ak-40
	A40	K-2,K-018,K-036
	A49	K-8,K-9,K-21,K-24,K-28,Ak-41,K-009,K-029,K-043
	5 steps to A3	K-59
	3 steps to A9	Ak-38
	1 step to A11	K-50
B	B1	K-3,K-4,K-6,K-11,K-12,K-13,K-14,K-16,K-18,K-19,K-20,K-23,K-25,K-27,
		K-29,K-30,K-32,K-46,K-47,K-48,K-52,K-53, K-54,K-56,K-57,K-62,K-63,
		K-005,K-012,K-013,K-015,K-023,K-030,K-031, K-048,K-051,K-056
		K-065,K-075,K-080
	B2	K-35,K-003,K-024,K-054,K-059
	B10	K-001
	B13	K-020
	1step to B1	K-55
	1step to B2	Ak-42
	1step to B3	K-58,K-073
C	C2	K-33,K-51
	C5	K-062
	C6	K-034,K-046
D	D5	K-17,K-45,K-60,K-61,K-007,K-014,K-044,K-049,K-060,K-061,K-070,K-074,K-076

Table 3.2. Number and proportion of individuals and number of haplotypes and unique haplotypes for the phylogenetic clades A, B, C, and D in Turkish dog samples and summary statistics of diversity measures for two Turkish breed.

	Number of individuals (proportion, %)	**Number of haplotypes (unique types)**	**Haplotype diversity**	**Mean number of pairwise difference**	**Nucleotide diversity**
Kangal					
Clade A	37 (35.2)	12 (2)	0.828±0.031	8.601±4.006	0.015±0.008
Clade B	50 (47.6)	6 (2)			
Clade C	5 (4.76)	3 (0)			
Clade D	13(12.3)	1 (0)			
Akbash					
Clade A	8 (88.8)	5 (1)	0.833±0.126	5.472±2.907	0.009±0.006
Clade B	1 (11.1)	1 (1)			

The Kangal dog presented the highest values of diversity measures, implying that it harbours a comparatively more variable gene pool.

3.2.2. Neighbor Joining (NJ) Tree of the Turkish Data

The phylogenetic relationships between haplotypes were constructed (Figure 3.6) using the neighbor-joining (NJ) method based on Kimura-2P model. The sequences of the wolves (downloaded from NCBI; accession numbers AF530561clW3, AF530562clW10, AF530563clW13, AF530564clW18, AF530565clW9 and AF530566clW15) were also used for comparison. Since same haplotypes were shared between some samples, the branches of the tree were collapsed and the newly obtained simplified NJ tree was reconstructed and presented in Figure 3.7. Furthermore, on this tree four clades (A to D) were indicated.

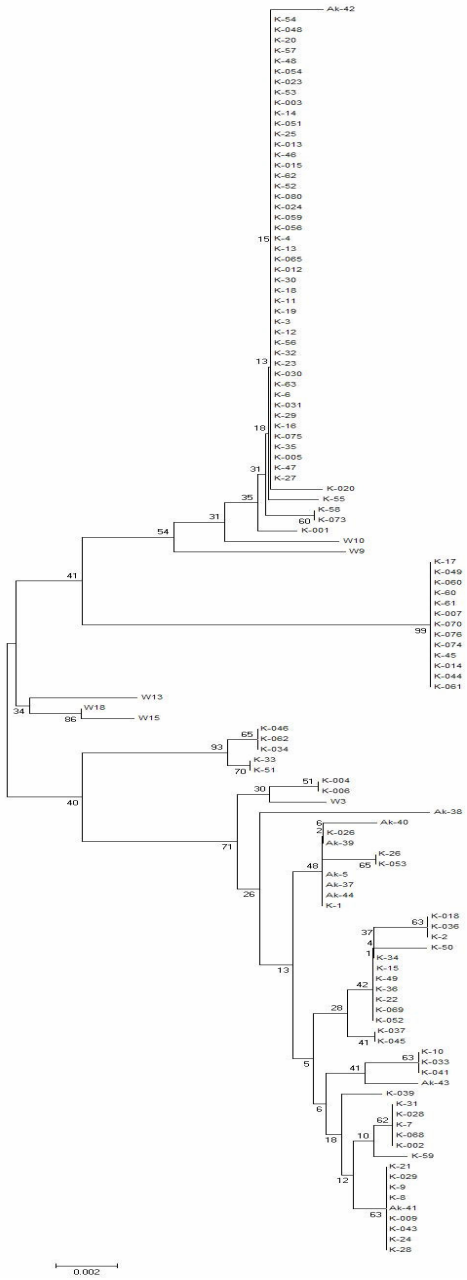


Figure 3.6. NJ tree of mtDNA control region sequences of all Turkish dog samples together with some wolf sequences.

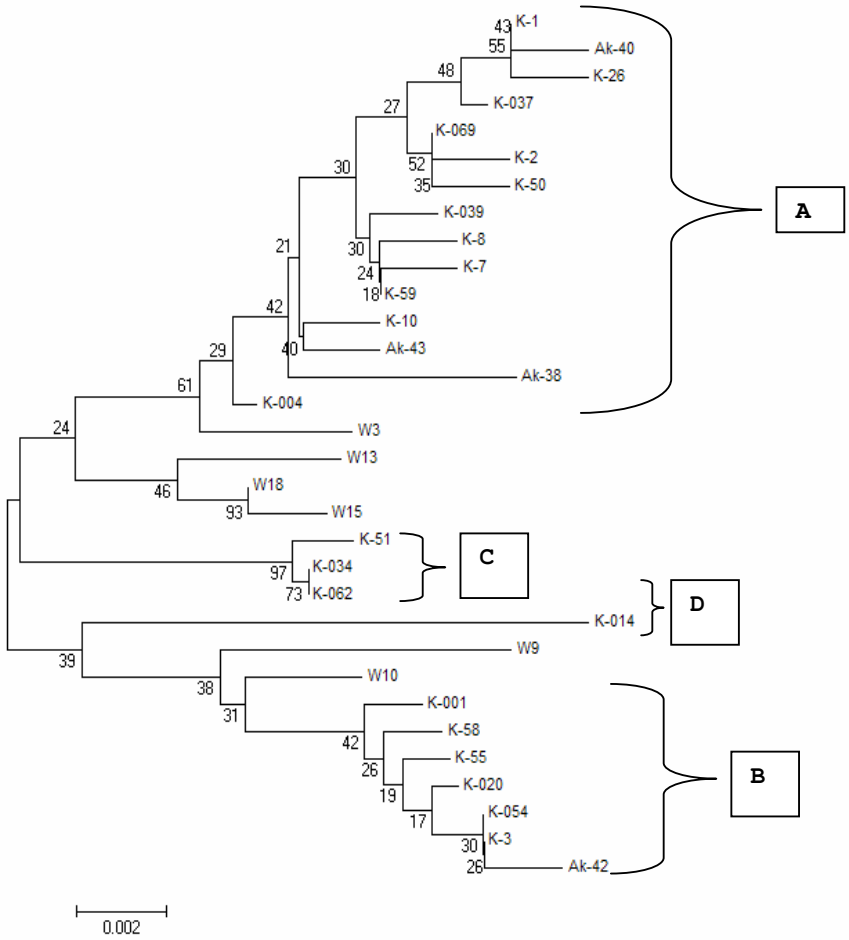


Figure 3.7. Collapsed NJ tree of mtDNA control region sequences of Turkish dog samples together with some wolf sequences.

In both of these trees, the letters in the sequence labels are; “K” for Kangal dog (e.g. K-10), “Ak” for Akbash dog (e.g. Ak-42), and “W” for wolf (e.g. W15) samples.

From the bootstrap values it can be seen that robustness of the topology is not very strong. Furthermore, all wolf sequences are relatively different than those of the Anatolian dogs.

3.2.3. Minimum Spanning Network

The relationships among mtDNA dog haplotypes can be studied in more detail in a minimum spanning network. It was calculated for each group using Arlequin software (http://anthropologie.unique.ch/arlequin). The haplotypes were represented by circles, the lines were used to connect the haplotypes (circles) that have close relationship with each other. In addition, on the lines mutational steps between the haplotypes were indicated. Two haplotypes connected with a line means, there is only one mutational step between them.

Firstly, the Minimum Spanning Network (MSN) was drawn for all haplogroups and types observed in Turkish dog samples and shown in Figure 3.8 (pp 44). In this figure the squares represent the missing haplotypes, which were not observed in the present study. Circle sizes are roughly proportional to the number of individuals they represent. For the unique haplotypes observed in Turkish dog samples, the letters ‘K’ and ‘Ak’ were used. ‘K’ represents the Kangal dog and ‘Ak’ represents the Akbash dog. And ‘n’ represents the sample size.

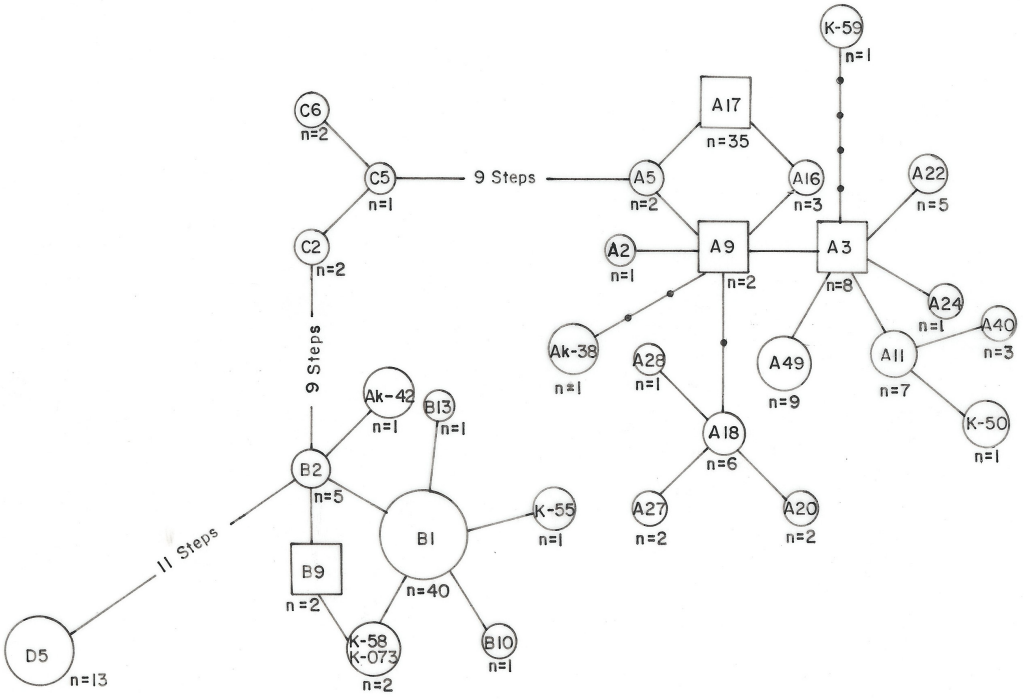


Figure 3.8. MSN for all haplogroups and types observed in Turkish dog samples.

From this figure, it can be seen that the most variable haplogroup is A and the least is D.

Next, the minimum spanning networks were drawn separately for haplotypes observed in Kangal and Akbash dogs. For these minimum spanning networks all the haplotypes observed previously in Kangal and Akbash dogs from the literature (n=10 for Kangal, n=11 for Akbash) were included, relatedness of haplotypes to the regions were indicated on the networks in paranthesis and presented in Figure 3.9 and 3.10 (pp 46 and 47).

In Figure 3.9, squares represent the missing haplotypes, which were not observed in the present study, ‘n’ represents the number of individual, circle sizes are roughly proportional to the number of individuals they represent. For the unique haplotypes observed in Kangal dog samples the letter ‘K’ was used.

In Figure 3.10, squares represent the missing haplotypes, which were not observed in the present study, ‘n’ represents the number of individual, circle sizes are roughly proportional to the number of individuals they represent. For the unique haplotypes observed in Akbash dog samples the letter ‘Ak’ was used.

In both figures numbers enclosed in paranthesis correspond to the region where same haplotypes were observed. 2: represents Southwest Asia; Afghanistan, Uzbekistan, Tadzikistan, Kazakstan, n=8, (SWA), 3: represents Middle East; Iran, Saudi Arabia, Dubai, Israel, Syria, n=38, (ME), 4: represents Scandinavia, n=49, (Scand.), 5: represents Europe; Britain, Continent, n=158, (Europe), and 6: represents East Asia; China, Japan, Korea, n=255, (EAsia). For example “A20 (3:5)” indicates that haplotype A20 was also found in Middle Eastern and European dog samples.

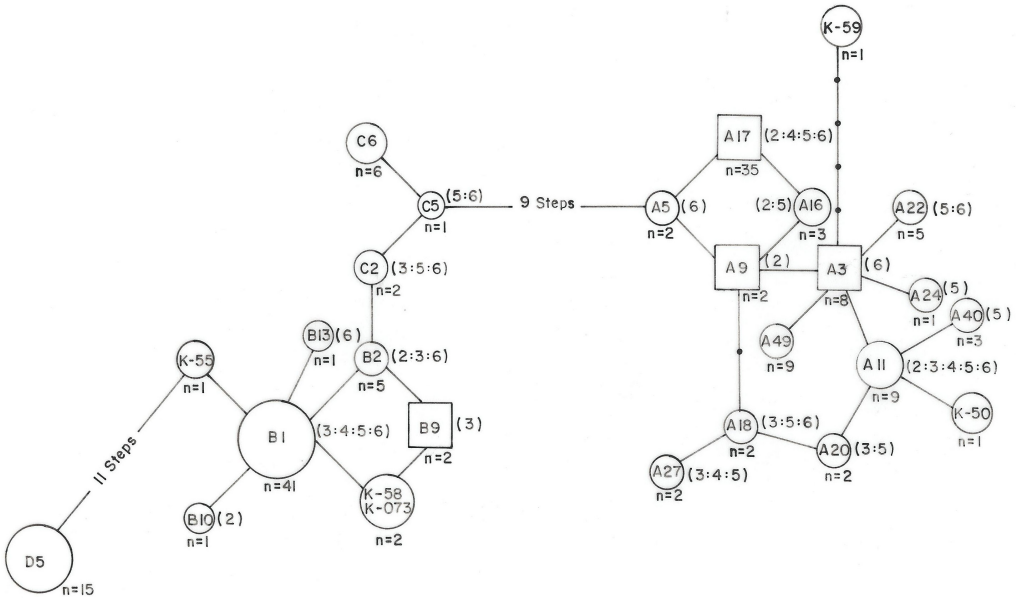


Figure 3. 9. MSN for haplotypes observed in Kangal dog samples.

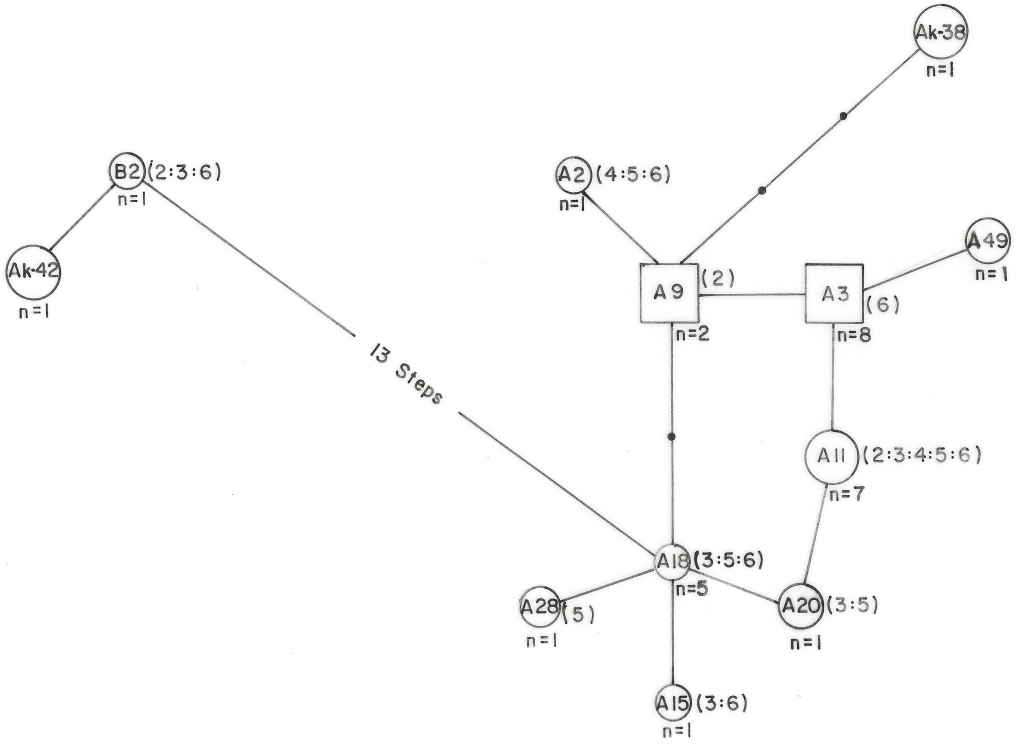


Figure 3.10. MSN for haplotypes observed in Akbash dog samples.

The results of the comparison of haplotypes with regions for both Kangal and Akbash dogs are as follows:

From the figure 3.9 it can be seen that there were 22 haplotypes observed in Kangal dog samples (n=115), 6 of them were in common with South West Asia (n=8), 7 of them were in common with Middle East (n=38), 4 of them were in common with Scandinavia (n=49), 12 of them were in common with Europe (n=158) and 11 of them were in common with East Asia (n=255).

From the figure 3.10 it can be seen that there were 12 haplotypes observed in Akbash dog samples (n=20), 3 of them were in common with South West Asia (n=8), 5 of them were in common with Middle East (n=38), 2 of them were in common with Scandinavia (n=49), 5 of them were in common with Europe (n=158) and 6 of them were in common with East Asia (n=255).

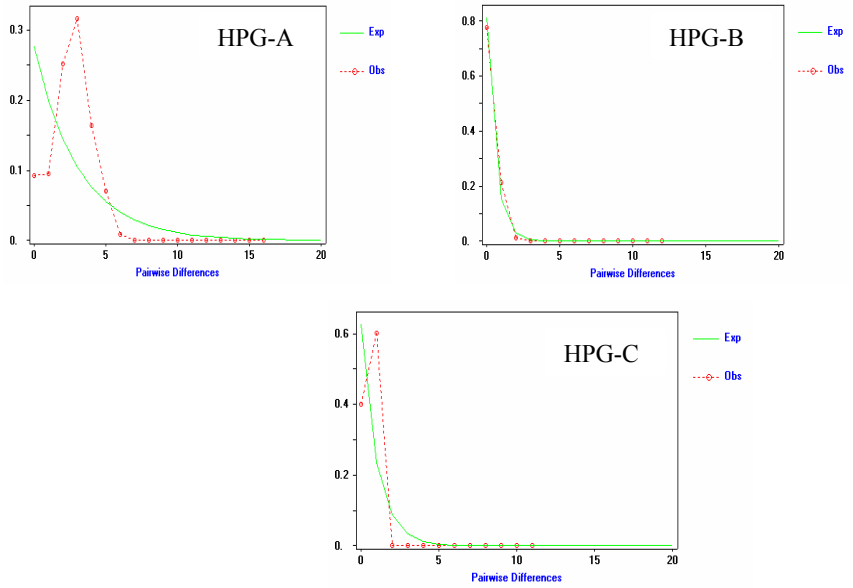
3.2.4. Mismatch Distributions

Nucleotide pairwise difference distributions also known as mismatch distributions were computed using the program DnaSP (http://www.ub.es/dnasp).

The mismatch distributions for the Turkish dogs observed in the present study were constructed for each clade separately. Mismatch distribution for clade D could not be computed as all of the Kangal dogs harboring the D haplotype were identical to each other.

The means of the distributions were different, suggesting different time spans for the origin and dispersal of each haplogroups. The mean difference values for haplogroup A was 2.615, for haplogroup B was 0.234 and for haplogroup C was 0.600. The mean difference value was considerably high in haplogroup A which indicated the oldest date of origin for this haplogroup, then C and B respectively.

A)



B)

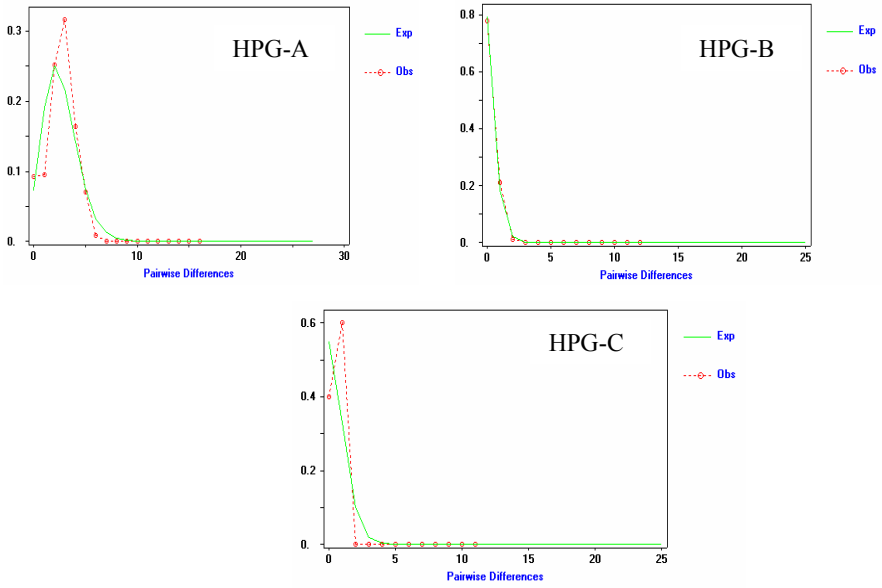


Figure 3.11. Observed and expected mismatch distributions for Turkish dog mtDNA control region sequences under both constant (upper graphs, A) and population expansion (lower graphs, B) expectations for haplogroups A, B and C.

In these graphs observed mismatch distributions were compared with the expected ones. In the upper graphs expected curves were drawn by assuming constant population size whereas in the lower garphs expected curves were drawn under the assumption of population growth. Because the fit between the observed and expected distributions was better in the lower half of the figures especially for haplogroup A, these distributions suggest that all haplogroups experienced population growth / expansion as expected for the domestic animals.

3.2.5. Neutrality Tests

For the detection of demographic history of the clades more sensitive tests can be applied on the clades A, B, C. Results are depicted in Table 3.3.

Table 3.3: Neutrality and diversity estimates for haplogroups from mtDNA control region sequences. Significant values in the data are indicated.

	HPG-A	**HPG-B**	**HPG-C**
Number of haplotypes	15	7	3
Nucleotide diversity, Pi, π	0.00453	0.00040	0.00103
Haplotype diversity (Hd)	0.908	0.223	0.600
Tajima's (1989) D	-0.899	-1.906 *	1.225
Fu & Li's (1993) F*	-1.44	-2.93	1.16
Fu & Li's (1993) D*	-1.38	-2.77	1.22
Fu's (1997) Fs	-5.50**	-6.17**	0.63
Raggedness (r)	0.064	0.360	0.400
τ	2.615	0.234	0.600

P<0.05 ; **P<0.01

Since in all clades nucleotide diversities were low but haplotype diversities were high, recent population growths were suggested. Perhaps more so for A haplogroup. However since the Tajima's D were negative only in haplogroups A and B but not in haplogroup C and since significant Fs values were observed in haplogroups A and B, perhaps haplogroup C did not experience population growth or because of the few haplotypes observed in Kangal dogs were not enough to detect the true demographic history of haplogroup C.

3.2.6. Turkish Data with the Data From Other Dogs

3.2.6.1. Population Pairwise Distances

To study the genetic similarity of Kangal and Akbash breeds to each other and to the dogs from different regions of Eurasia, pairwise D_A distances (Nei and Li,1979) between Kangal, Akbash and the dogs from different geographic regions were calculated and shown in the upper triangle of table 3.4. In the lower half of the same table (table 3.4) F_{ST} values together with their significances (+) were given. In this table Kangal and Akbash dogs examined in the present study are pooled with the ones studied by Savolainen et al., (2002).

Table 3.4: Pairwise estimates of F_{ST} (below diagonal) and D_A genetic distances (above diagonal) among nine groups.

	Akbas	**Kangal**	**SWAsia**	**MEast**	**Scand.**	**Europe**	**EAsia**	**Siberia**	**India**
Akbash	*0.000*	**2.464**	2.916	0.957	2.941	0.166	0.21	0.355	0.352
Kangal	**0.224+**	*0.000*	0.088	0.432	0.605	1.575	1.43	1.639	2.769
SWAsi	0.366+	0.004-	*0.000*	0.121	1.147	2.141	1.90	2.428	3.495
MEast	0.119+	0.047+	0.015-	*0.000*	1.193	0.592	0.50	0.830	1.475
Scand.	0.268+	0.063+	0.106	0.120+	*0.000*	1.970	1.98	2.023	3.209
Europe	0.018-	0.174+	0.254	0.082+	0.218	*0.000*	0.05	0.056	0.508
EAsia	0.021-	0.157+	0.214	0.066+	0.211	0.008+	*0.00*	0.040	0.506
Siberia	0.056+	0.158+	0.255	0.097+	0.190	0.009-	0.00	*0.000*	0.493
India	0.073+	0.236+	0.365	0.161+	0.269	0.067+	0.05	0.067	*0.000*

Comparison of the values of Kangal and Akbash breeds it can be seen that the Kangal and Akbash breeds are significantly different from each other (F_{ST}=0.224, P<0.05) and the genetic distance between them is quite large (2.464).

3.2.6.2. Neighbor Joining (NJ) Tree of the Dogs from Different Geographic Regions

Based on the genetic distances, genetic similarities between the compared groups were displayed on the constructed NJ tree (Figure 3.12).

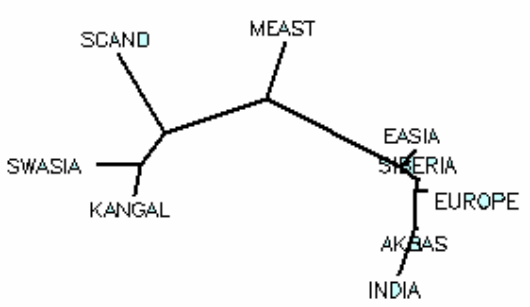


Figure 3.12. NJ tree based on D_A distances between nine groups.

In the next analysis Indian and South West Asian samples were excluded from the analysis since they have lower number of individuals (n_{SWAsia}= 8, n_{India} =13) compared to other regions. And the result was shown in Figure 3.13.

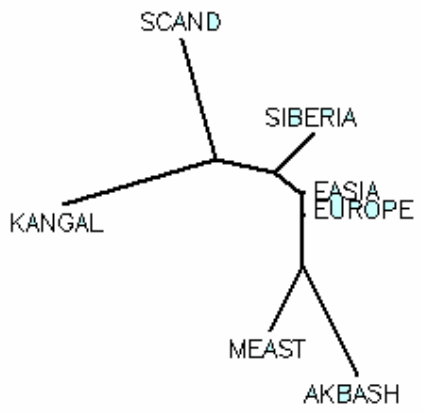


Figure 3.13. NJ tree based on D_A distances between seven groups.

From these figures it can be easily seen that Kangal and Akbash dogs were on the opposite ends of the tree. Kangal dogs are more similar to the Scandinavian and South West Asian (Afghanistan, Uzbekistan, Tadzikistan, Kazakstan) dogs whereas Akbash dogs exhibited high similarity to European (Britain, Continent), Indian, Siberian, East Asian (China, Japan, Korea) and Middle Eastern (Iran, Saudi Arabia, Dubai, Israel, Syria) dogs. Hence, although the samples were collected from the Anatolia, genetic similarity between the two breeds were low.

3.2.6.3. Analysis of Molecular Variance (AMOVA) of Different Dog Groups

In order to see how the genetic variation was partitioned between groups, AMOVA was employed. Three groups were formed, namely Group I (Kangal, Scandinavia, Southwest Asia) , Group II (Middle East) and Group III (Akbash, Europe, East Asia, Siberia, India). From the analysis it was revealed that 83.74% of the total variation was partitioned within populations and 13.90% was partitioned among groups. The difference among groups was higher than that of among

populations within the groups. There was a significant difference among the groups (ϕ_{CT}=0.13898; P=0.0078) (Table 3.5). However, the differences between the dogs within the groups were not significant (p>0.05).

Table 3.5. AMOVA of different dog groups.

Source of variation	d.f.	Variance components	Percentage of variation	Fixation index
Among groups	2	0.61270	13.90	0.13898 ** ϕ_{CT}
Among populations within groups	6	0.10410	2.36	0.02742 ns ϕ_{SC}
Within populations	671	3.69181	83.74	0.16259 ϕ_{ST}
Total	679	4.40861		

** P<0.01, ns P>0.05

CHAPTER IV

DISCUSSION

A comprehensive genetic study completed by Savolainen et al. (2002), which includes all major dog populations worldwide, grouped all dogs into 6 haplogroups (A to F) with respect to their maternal inheritance. The frequencies of haplogroups A, B and C were similar in all regions (see Figure 5.1) and there was no division of the main morphologic types of dogs, all of them had major haplogroups (A, B, C). In addition, based on the larger genetic variation in East Asia than in other regions, an East Asian origin for the domestic dog was suggested. Moreover, it was suggested that a single gene pool containing major haplogroups A, B and C was formed in East Asia and then dogs dispersed worldwide, present day dog breeds were formed by selection from the local pools brought from East Asia. Whereas, based on the Savolainen et al. (2002), it can be concluded that D, E and F haplogroups were introduced to the dog gene pool locally from the wolves after the dispersion from the East Asia.

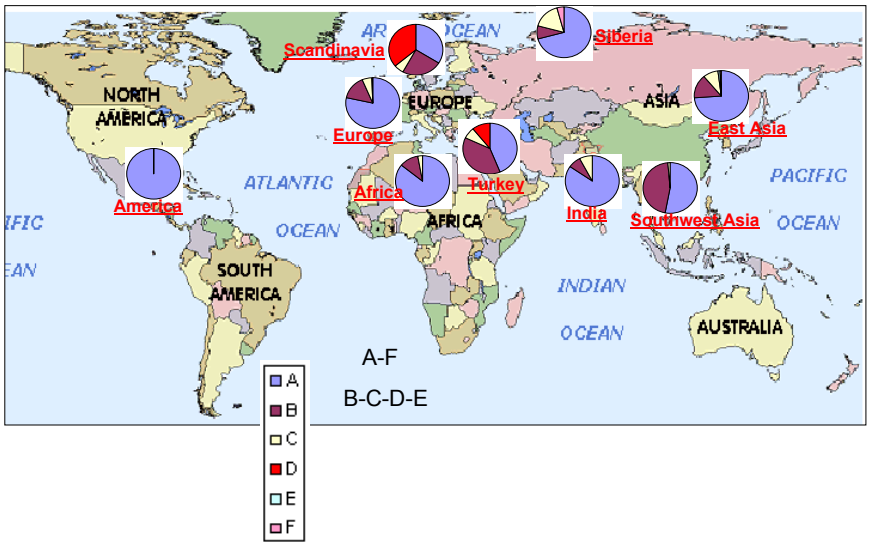


Figure 4.1. Worldwide distributions of haplotypes observed in Savolainen et al.'s (2002) study.

In the present study, three major haplogroups A, B and C were also observed in Turkish dog samples. For the construction of NJ tree, the sequences of wolves were also used, and it was detected that all wolf sequences are relatively different from than those of Turkish dogs. That can be because of the Chinese origin for all wolf samples used in the present study. Whereas, in Savolainen et al's (2002) study Clade A included 3 wolf haplotypes found in China and Mongolia and Clade B contained 3 wolf haplotypes 2 found in East Europe and 1 in Afghanistan. From the mismatch analysis, the oldest date of origin for haplogroup A, then C and B based on their mean difference values was indicated in Turkish dog samples. The mismatch distribution graphs of haplogroups under population growth expectation indicated that all haplogroups experienced population expansion as expected for the domestic animals. This case was also recovered from the neutrality tests except for haplogroup C (Tajima's D was not negative and significant F_S values were not observed). It may be because of the few haplotypes observed in haplogroup C which were not enough

to detect the true demographic history. In the previous studies, relatively low variability observed in haplogroup C was also indicated (Savolainen et al., 2002; van Asch et al.,2005).

The results which obtained from the comparison of Kangal and Akbash gene pools are as follows:

Kangal dogs presented the highest values of diversity measures implying that it harbours a comparatively more variable gene pool whereas Akbash dogs presented low levels of diversity, which could be explained by small sample size.

In Kangal dogs (n=105), there were 3 major haplogroups (A, B and C), 4 new haplotypes (two of them are belonging to Clade A and two of them are belonging to Clade B) and a rare haplogroup D. The Kangal dogs (n=13) harbouring the D haplogroup had the single type, D5 which was not observed in any other breed until now. They were all D haplotypes (D1-D4) were previously described in eight Scandinavian breeds (n=18), two Kangal dogs (D5) (n=10), seven Serra da Estrela Mountain dogs (ID) (Portuguese, n=34) and one Galgo Espanol dog (D6) (Spain). D5 was 5 steps apart from Scandinavian D1, D2, D3 and D4, 1 step apart from Portuguese ID and 2 steps apart from Spanish D6 (Figure 5.2).

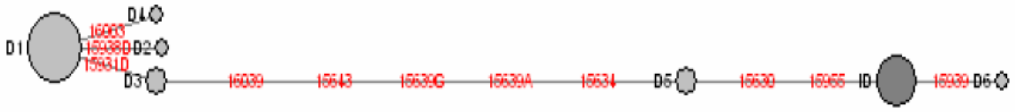


Figure 4.2. MSN network of dog mtDNA haplotypes belonging to haplogroup D (taken from van Asch et al., 2005 study).

Hence, with respect to D haplogroup since the highest diversity is in Scandinavian dogs and more than one breed, 8 breeds exhibited 4 D haplogroups it can be said that among the studied dogs Scandinavians are the closest to the introduction of D haplogroup into dog gene pool. Because of the fact that D5 (Kangal type) is the one which is closest to the Scandinavian dogs among the other dogs harboring the D haplogroup (see figure 5.1), perhaps Kangals are representing the second breed which is again relatively close to the D haplogroup introduction center.

In Akbash dogs (n=9), there were 2 haplogroups A and B and 2 new haplotypes (one was belonging to Clade A and the other was belonging to Clade B). Haplogroup C and relatively rare haplogroup D was not detected in Akbash dogs (n=9 in the present study, n=11 in Savolainen et al.'s 2002 and van Asch et al.'s 2005 study). Total absence of D in Akbash dogs suggested that these two dogs descended maternally from different origins along the evolutionary history of dogs.

From the comparison of genetic similarity of Kangal and Akbash dogs to each other and the dogs from different regions of Eurasia indicated that Kangal and Akbash dogs are significantly different from each other, the genetic distance between them is quite large and both of them are equally distant from Middle Eastern dog samples (see figure 3.11 in the result part). It was also indicated that Kangal dogs are more close to Scandinavian and South West Asian dogs whereas Akbash dogs are close to European, East Asian, Indian and Siberian dogs as was proved by the results of AMOVA and Akbash but not Kangal dog was relatively close to Middle Eastern dogs (see figure 3.12 in the result part). Even for Kangal and Akbash dogs are geographically close they revealed different histories as was suggested by the trees and the presence of D haplotype only in Kangal. The similar observation was made in Portugal : out of 4 native dog breeds only one (Serra da Estrela Mountain Dog) had 20 % D haplotype (van Asch et al., 2005).

Domestic animals have joint history of evolution with the humans for the approximately 10 000 years, dogs perhaps had longer joint history 15 000-40 000 years B.P as suggested by Savolainen et al., 2002. If the origin of domestication for the dogs is Eastern Asia was assumed their migration to the west might have been through more than a single route. To provide an alternative hypothesis for their migration routes, it is necessary to study the human historical / prehistorical records.

At the latest glacial, around 18 000 years ago, Northern and Central Europe were covered with glaciers. By the retreat of ices North Eurasian human migration might have brought D haplogroup and distributed mainly to Scandinavia. This route might have left the imprints in Eastern Central Anatolia, Kangal by the migration branch through the Caucasus.

A recent study on the human admixture proportion indicates a 52% migration from Middle East which covers Turkey (Dupanloup et al., 2004). Presence of D haplogroup in Kangal and the human gene pool similarity between Scandinavia and Middle East may have some common prehistorical reason. Alternatively, Kangal dogs might have been brought to Anatolia along with the migrations of the nomadic Turks from Central Asia as was suggested by Karadağ (2002). The dog of nomadic Turks might be harboring D haplotype(s) from the introduction site of D haplogroup. Karadağ (2002)'s suggestion was based on the name "kangal" and on the appearances of the dogs resembling Kangal dogs in Turkestan today. Furthermore, South West Asian dogs were found to be the closest to the Kangal dogs in the present study may be a support for this hypothesis.

Nomadic Turks were sheep breeders they initially settled on the Eastern, Central Eastern parts of the Anatolia and today Kangal dogs are known as the shepherd dogs. If the second scenario is the valid one, the low diversity for D in Kangal dogs could also be explaind due to long distance of migration. On the other

hand , based on the mtDNA sequences Akbash dogs are perhaps following a Southern route through Asia.

These alternative scenarios can be tested by the samples to be collected from Caucasus and along the route of Nomadic Turks, as well as the wolf samples to be collected from the same places and North Eurasia.

The present study strongly supports the main argument that Kangal and Akbash dogs must be given different breed names and must be conserved separately. Although, mtDNA tells us the maternal history of the populations, one of the previous study carried on the samples of these two dogs based on 100 canine microsatellites also revealed the distinctness of these two dogs (Altunok et al., 2005).

CHAPTER V

CONCLUSIONS

Results of the present study indicated that there is a significant genetic differentiation among the Kangal and Akbash dogs. Even though they are geographically close, the genetic similarity between the two dogs are low. Presence of D haplogroup in Kangal and not in Akbash dogs suggests that these two dogs might have different origins along the evolutionary history of dogs. From the result of statistical analysis it is detected that Kangal dogs are genetically close to Scandinavian and Southwest Asian dogs whereas Akbash dogs are close to European, East Asian dogs. The distinctness of these two breeds was in accordance with the study based on 100 canine microsatellites (Altunok et al., 2005). Therefore, Kangal and Akbash dogs must be recognized separately and an urgent careful breeding program must be carried out within these breeds because their numbers are alarmingly decreasing. For future studies there are important things to take into account. These are as follows:

The number of studied Turkish dog samples both in the literature and present study needs to be improved. For the statistical confidence of the results, the numbers of samples should be at least 30, more sampling from Turkish dogs is necessary and for comparison feral dogs and Turkish greyhound samples should be included and

the number of Akbash dog samples should be increased. For understanding the history of Turkish dogs better, samples should be collected from Caucasus and along the route of Nomadic Turks. The incidence of D haplotype must also be searched from these areas which can shed light on the understanding of origin of Kangal dogs. All the samples should be analysed using more and different types of molecular markers; SNPs, microsatellites, mtDNA and Y-chromosome to get a better picture and results should be evaluated comparatively and carefully for developing conservation strategies. For enhancing the results, collaborative studies with other geographic regions as well as with other disciplines (e.g. archaeologists, anthropologists, geneticist and statisticians) are necessary.

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APPENDIX A: Table of Breed Informations

Table A: A list presenting all dogs used in the present study from the literature (Savolainen et al., 2002). Columns give haplotype, breed, different regional origins, region. E/W gives an origin from the eastern (E) or the western (W) parts of the world.

Haplotype	Breed	Subregion	Region	E/W	Total
TURKEY					
A2	Anatholian Shepherd	Turkey	Southwest Asia	W	1
A11	Akbash	Turkey	Southwest Asia	W	1
A11	Akbash	Turkey	Southwest Asia	W	1
A11	Akbash	Turkey	Southwest Asia	W	1
A11	Akbash	Turkey	Southwest Asia	W	1
A11	Akbash	Turkey	Southwest Asia	W	1
A11	Akbash	Turkey	Southwest Asia	W	1
A11	Akbash	Turkey	Southwest Asia	W	1
A11	Kangal	Turkey	Southwest Asia	W	1
A11	Kangal	Turkey	Southwest Asia	W	1
A15	Akbash	Turkey	Southwest Asia	W	1
A16	Anatholian Shepherd	Turkey	Southwest Asia	W	1

Table A cont'd…

A17	Anatholian Pharia	Turkey	Southwest Asia	W	1
A18	Akbash	Turkey	Southwest Asia	W	1
A20	Akbash	Turkey	Southwest Asia	W	1
A49	Kangal	Turkey	Southwest Asia	W	1
B1	Kangal	Turkey	Southwest Asia	W	1
B2	Akbash	Turkey	Southwest Asia	W	1
C6	Kangal	Turkey	Southwest Asia	W	1
C6	Kangal	Turkey	Southwest Asia	W	1
C6	Kangal	Turkey	Southwest Asia	W	1
C6	Kangal	Turkey	Southwest Asia	W	1
D5	Kangal	Turkey	Southwest Asia	W	1
D5	Kangal	Turkey	Southwest Asia	W	1
SWASIA					
A11	Taigan	Uzbekistan	Southwest Asia	W	1
A12	Afghan	Afghanistan	Southwest Asia	W	1
A19		Tadzikistan	Southwest Asia	W	1
B2	Tazi	Kazakstan	Southwest Asia	W	1
B2	Afghan	Afghanistan	Southwest Asia	W	1
B2	Afghan	Afghanistan	Southwest Asia	W	1
B2	Afghan	Afghanistan	Southwest Asia	W	1
B2	Afghan	Afghanistan	Southwest Asia	W	1
MIDDLE EAST					
A11		Iran	Middle East	E	1
A11	Canaan Dog	Israel	Middle East	E	1
A11	Canaan Dog	Israel	Middle East	E	1
A11	Canaan Dog	Israel	Middle East	E	1

Table A cont'd…

A11	Canaan Dog	Israel	Middle East	E	1
A11	Canaan Dog	Israel	Middle East	E	1
A11	Canaan Dog	Israel	Middle East	E	1
A15	Saluki	Saudi arabia	Middle East	E	1
A18		Iran	Middle East	E	1
A19		Iran	Middle East	E	1
A19		Iran	Middle East	E	1
A19		Iran	Middle East	E	1
A19		Iran	Middle East	E	1
A19	Canaan Dog	Israel	Middle East	E	1
A20	Canaan Dog	Israel	Middle East	E	1
A20	Canaan Dog	Israel	Middle East	E	1
A21		Iran	Middle East	E	1
A25		Iran	Middle East	E	1
A25		Iran	Middle East	E	1
A26	Saluki	Saudi arabia	Middle East	E	1
A27		Iran	Middle East	E	1
A43	Saluki	Saudi arabia	Middle East	E	1
B1		Iran	Middle East	E	1
B1	Saluki	Saudi arabia	Middle East	E	1
B2	Saluki	Dubai	Middle East	E	1
B2		Iran	Middle East	E	1
B2		Iran	Middle East	E	1
B2	Canaan Dog	Israel	Middle East	E	1
B2	Canaan Dog	Israel	Middle East	E	1
B2	Canaan Dog	Israel	Middle East	E	1
B2	Canaan Dog	Israel	Middle East	E	1
B2	Canaan Dog	Israel	Middle East	E	1
B2	Saluki	syria	Middle East	E	1
B6	Canaan Dog	Israel	Middle East	E	1
B6	Canaan Dog	Israel	Middle East	E	1
B9	Canaan Dog	Israel	Middle East	E	1
B9	Canaan Dog	Israel	Middle East	E	1
C2		Iran	Middle East	E	1
SCANDINAVIA					
A1	Buhund	Scandinavia	Europe	W	1
A1	Buhund	Scandinavia	Europe	W	1
A2	Finnish Spitz	Scandinavia	Europe	W	1
A2	Icelanding Sheep Dog	Scandinavia	Europe	W	1
A2	Icelanding Sheep Dog	Scandinavia	Europe	W	1
A2	Norrbottenspets	Scandinavia	Europe	W	1

Table A cont'd…

A11	Buhund	Scandinavia	Europe	W	1
A11	Buhund	Scandinavia	Europe	W	1
A11	Icelanding Sheep Dog	Scandinavia	Europe	W	1
A11	Icelanding Sheep Dog	Scandinavia	Europe	W	1
A11	Norwegian Elkhound	Scandinavia	Europe	W	1
A11	Norwegian Elkhound	Scandinavia	Europe	W	1
A17	Finnish Lapphund	Scandinavia	Europe	W	1
A17	Finnish Lapphund	Scandinavia	Europe	W	1
A17	Icelanding Sheep Dog	Scandinavia	Europe	W	1
A27	Lundehund	Scandinavia	Europe	W	1
A27	Lundehund	Scandinavia	Europe	W	1
B1	Buhund	Scandinavia	Europe	W	1
B1	Finnish Spitz	Scandinavia	Europe	W	1
B1	Finnish Spitz	Scandinavia	Europe	W	1
B1	Finnish Spitz	Scandinavia	Europe	W	1
B1	Finnish Spitz	Scandinavia	Europe	W	1
B1	Finnish Spitz	Scandinavia	Europe	W	1
B1	Finnish Spitz	Scandinavia	Europe	W	1
B1	Norrbottenspets	Scandinavia	Europe	W	1
B1	Norrbottenspets	Scandinavia	Europe	W	1
B1	Norrbottenspets	Scandinavia	Europe	W	1
B3	Finnish Spitz	Scandinavia	Europe	W	1
C3	Jamthund	Scandinavia	Europe	W	1
C3	Jamthund	Scandinavia	Europe	W	1
C3	Stövare Finsk	Scandinavia	Europe	W	1
D1	Finnish Lapphund	Scandinavia	Europe	W	1
D1	Finnish Lapphund	Scandinavia	Europe	W	1
D1	Finnish Lapphund	Scandinavia	Europe	W	1
D1	Finnish Lapphund	Scandinavia	Europe	W	1
D1	Jamthund	Scandinavia	Europe	W	1
D1	Jamthund	Scandinavia	Europe	W	1
D1	Lappsk Vallhund	Scandinavia	Europe	W	1
D1	Norwegian Elkhound	Scandinavia	Europe	W	1
D1	Norwegian Elkhound	Scandinavia	Europe	W	1
D1	Norwegian Elkhound	Scandinavia	Europe	W	1
D1	Swedish Lapphund	Scandinavia	Europe	W	1
D1	Swedish Lapphund	Scandinavia	Europe	W	1
D1	Swedish Lapphund	Scandinavia	Europe	W	1
D1	Swedish Lapphund	Scandinavia	Europe	W	1

Table A cont'd…

D2	Finnish Spitz	Scandinavia	Europe	W	1
D3	Jamthund	Scandinavia	Europe	W	1
D3	Lappsk Vallhund	Scandinavia	Europe	W	1
D4	Lappsk Vallhund	Scandinavia	Europe	W	1
EUROPE					
A1	Border Collie	Britain	Europe	W	1
A1	Border Collie	Britain	Europe	W	1
A1	Border Collie	Britain	Europe	W	1
A1	Border Collie	Britain	Europe	W	1
A1	Collie	Britain	Europe	W	1
A1	Collie	Britain	Europe	W	1
A1	Working Kelpie	Britain	Europe	W	1
A2	Pointer	Britain	Europe	W	1
A2	Setter English	Britain	Europe	W	1
A2	Setter English	Britain	Europe	W	1
A2	Terrier West Highland	Britain	Europe	W	1
A2	Leonberger	Continent	Europe	W	1
A2	Leonberger	Continent	Europe	W	1
A2	Leonberger	Continent	Europe	W	1
A2	Leonberger	Continent	Europe	W	1
A2	Leonberger	Continent	Europe	W	1
A11	Fox Terrier	Britain	Europe	W	1
A11	Pointer	Britain	Europe	W	1
A11	Terrier Border	Britain	Europe	W	1
A11	Terrier Border	Britain	Europe	W	1
A11	Terrier Kerry Blue	Britain	Europe	W	1
A11	Whippet	Britain	Europe	W	1
A11	Basset Griffon	Continent	Europe	W	1
A11	Borzoi	Continent	Europe	W	1
A11	Drever	Continent	Europe	W	1
A11	Drever	Continent	Europe	W	1
A11	Gos D'atura Catalan	Continent	Europe	W	1
A11	Mudi	Continent	Europe	W	1
A11	Papillon	Continent	Europe	W	1
A11	Pyreneean Mastiff	Continent	Europe	W	1
A11	Rottweiler	Continent	Europe	W	1
A11	Rottweiler	Continent	Europe	W	1
A11	Rottweiler	Continent	Europe	W	1
A11	Rottweiler	Continent	Europe	W	1
A11	Rottweiler	Continent	Europe	W	1
A11	Rottweiler	Continent	Europe	W	1
A11	Rottweiler	Continent	Europe	W	1
A11	Rottweiler	Continent	Europe	W	1

Table A cont'd…

A11	Vastgötaspets	Continent	Europe	W	1
A11	Vastgötaspets	Continent	Europe	W	1
A16	New Foundland	Britain	Europe	W	1
A16	Retriever Golden	Britain	Europe	W	1
A16	Retriever Golden	Britain	Europe	W	1
A16	Retriever Golden	Britain	Europe	W	1
A16	Retriever Labrador	Britain	Europe	W	1
A16	Retriever Labrador	Britain	Europe	W	1
A16	Retriever Labrador	Britain	Europe	W	1
A17	Cav.K.Charles Spaniel	Britain	Europe	W	1
A17	Norfolk Terrier	Britain	Europe	W	1
A17	Retriever Chesapeake Bay	Britain	Europe	W	1
A17	Retriever Flatcoated	Britain	Europe	W	1
A17	Retriever Labrador	Britain	Europe	W	1
A17	Retriever Labrador	Britain	Europe	W	1
A17	Retriever Labrador	Britain	Europe	W	1
A17	Retriever Labrador	Britain	Europe	W	1
A17	Retriever Labrador	Britain	Europe	W	1
A17	Retriever Labrador	Britain	Europe	W	1
A17	German Shepherd	Continent	Europe	W	1
A17	German Shepherd	Continent	Europe	W	1
A17	German Shepherd	Continent	Europe	W	1
A17	Leonberger	Continent	Europe	W	1
A17	Pug	Continent	Europe	W	1
A17	Pug	Continent	Europe	W	1
A18	Beagle	Britain	Europe	W	1
A18	Border Collie	Britain	Europe	W	1
A18	Setter English	Britain	Europe	W	1
A18	Setter English	Britain	Europe	W	1
A18	Beauceron	Continent	Europe	W	1
A18	Bracco Italiano	Continent	Europe	W	1
A18	Bracco Italiano	Continent	Europe	W	1
A18	Dalmatian	Continent	Europe	W	1
A18	Maltese	Continent	Europe	W	1
A18	Maremmano Abruzzese	Continent	Europe	W	1
A18	Poodle	Continent	Europe	W	1
A18	Pyreneisk Vallhund	Continent	Europe	W	1
A19	German Shepherd	Continent	Europe	W	1
A19	German Shepherd	Continent	Europe	W	1
A19	German Shepherd	Continent	Europe	W	1
A19	German Shepherd	Continent	Europe	W	1
A19	German Shepherd	Continent	Europe	W	1

Table A cont'd…

A19	German Shepherd	Continent	Europe	W	1
A19	German Shepherd	Continent	Europe	W	1
A19	German Shepherd	Continent	Europe	W	1
A19	German Shepherd	Continent	Europe	W	1
A19	German Shepherd	Continent	Europe	W	1
A19	Keeshond	Continent	Europe	W	1
A19	Keeshond	Continent	Europe	W	1
A20	Pointer	Britain	Europe	W	1
A20	Terrier Irish Soft Coated Wheaten	Britain	Europe	W	1
A20	Maltesian Phraohound	Continent	Europe	W	1
A20	Dachshound Wirehaired	Continent	Europe	W	1
A20	Dachshound Wirehaired	Continent	Europe	W	1
A20	Dachshund	Continent	Europe	W	1
A20	Dachshund	Continent	Europe	W	1
A20	Podenko Ibicenco	Continent	Europe	W	1
A20	Tervuerense	Continent	Europe	W	1
A22	English Setter	Britain	Europe	W	1
A22	Irish Wolfound	Britain	Europe	W	1
A22	Scottish Deerhound	Britain	Europe	W	1
A22	Boxer	Continent	Europe	W	1
A22	St Bernhard	Continent	Europe	W	1
A22	St Bernhard	Continent	Europe	W	1
A23	Borzoi	Continent	Europe	W	1
A24	Irish Wolfound	Britain	Europe	W	1
A24	Irish Wolfound	Britain	Europe	W	1
A24	Irish Wolfound	Britain	Europe	W	1
A24	Great Pyrenese	Continent	Europe	W	1
A24	Pyrene	Continent	Europe	W	1
A24	Pyreneer	Continent	Europe	W	1
A26	Wirehaired Foxterrier	Britain	Europe	W	1
A27	Maremmano Abruzzese	Continent	Europe	W	1
A27	Maremmano Abruzzese	Continent	Europe	W	1
A28	Beagle	Britain	Europe	W	1
A28	Cav.K.Charles Spaniel	Britain	Europe	W	1
A30	Hamiltonstövare	Continent	Europe	W	1
A30	Hamiltonstövare	Continent	Europe	W	1
A34	Galgo Espanol	Continent	Europe	W	1
A36	Pyreneer	Continent	Europe	W	1

Table A cont'd…

A40	Puli	Continent	Europe	W	1
A66	Cav.K.Charles Spaniel	Britain	Europe	W	1
A70	Shetland Sheepdog	Britain	Europe	W	1
A70	Shetland Sheepdog	Britain	Europe	W	1
A70	Shetland Sheepdog	Britain	Europe	W	1
B1	Otterhound	Britain	Europe	W	1
B1	Retriever Golden	Britain	Europe	W	1
B1	Retriever Golden	Britain	Europe	W	1
B1	Retriever Golden	Britain	Europe	W	1
B1	Retriever Golden	Britain	Europe	W	1
B1	Retriever Labrador	Britain	Europe	W	1
B1	Terrier Yorkshire	Britain	Europe	W	1
B1	Working Kelpie	Britain	Europe	W	1
B1	Dachsund	Continent	Europe	W	1
B1	Dachsund	Continent	Europe	W	1
B1	Dobberman Pinscher	Continent	Europe	W	1
B1	Dobberman Pinscher	Continent	Europe	W	1
B1	Dobberman Pinscher	Continent	Europe	W	1
B1	Finsk Stövare	Continent	Europe	W	1
B1	Groenedael	Continent	Europe	W	1
B1	Kooikerhondje	Continent	Europe	W	1
B1	Weimaraner	Continent	Europe	W	1
B3	Kuvasz	Continent	Europe	W	1
B6	Dachound	Continent	Europe	W	1
B6	Dachshound Wirehaired	Continent	Europe	W	1
B6	Dutch Sheperd	Continent	Europe	W	1
B6	Dutch Sheperd	Continent	Europe	W	1
B7	D\S Gardshund	Britain	Europe	W	1
B8	Retriever Flatcoated	Britain	Europe	W	1
B12	Affenpinscher	Continent	Europe	W	1
C1	Pittbull	Britain	Europe	W	1
C1	German Shepherd	Continent	Europe	W	1
C1	German Shepherd	Continent	Europe	W	1
C1	German Shepherd	Continent	Europe	W	1
C2	Terrier West Highland	Britain	Europe	W	1
C3	Terrier Airedale	Britain	Europe	W	1
C3	Riesenschnauzer	Continent	Europe	W	1
C3	Riesenschnauzer	Continent	Europe	W	1
C5	Sarplaninac	Continent	Europe	W	1
D6	Galgo Espanol	Continent	Europe	W	1

Table A cont'd…

EAST ASIA					
A1	Chow-Chow	China	East Asia	E	1
A1	Chow-Chow	China	East Asia	E	1
A1	Chow-Chow	China	East Asia	E	1
A1	Chow-Chow	China	East Asia	E	1
A2	Iki	Japan	East Asia	E	1
A2	Iki	Japan	East Asia	E	1
A2	Iki	Japan	East Asia	E	1
A3	Chow-Chow	China	East Asia	E	1
A3	Chow-Chow	China	East Asia	E	1
A3	Chow-Chow	China	East Asia	E	1
A3	Shar-Pei	China	East Asia	E	1
A3		China	East Asia	E	1
A3		China	East Asia	E	1
A5	Tibetan Terrier	China	East Asia	E	1
A5	Tibetan Terrier	China	East Asia	E	1
A5	Tibetan Terrier	China	East Asia	E	1
A5	Tibetan Terrier	China	East Asia	E	1
A5	Tibetan Terrier	China	East Asia	E	1
A5	Ryukyu	Japan	East Asia	E	1
A6	Thai Ridgeback	Southeast Asia	East Asia	E	1
A7	Thai Ridgeback	Southeast Asia	East Asia	E	1
A8	Thai Ridgeback	Southeast Asia	East Asia	E	1
A11	Chow-Chow	China	East Asia	E	1
A11	Pekingese	China	East Asia	E	1
A11	Pekingese	China	East Asia	E	1
A11	Pekingese	China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1

Table A cont'd…

A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11		China	East Asia	E	1
A11	Akita	Japan	East Asia	E	1
A11	Mikawa	Japan	East Asia	E	1
A11	Mikawa	Japan	East Asia	E	1
A11	Shiba	Japan	East Asia	E	1
A11	Shiba	Japan	East Asia	E	1
A11	Shikoku	Japan	East Asia	E	1
A11	Jindo	Korea	East Asia	E	1
A11	Jindo	Korea	East Asia	E	1
A11	Jindo	Korea	East Asia	E	1
A11	Jindo	Korea	East Asia	E	1
A11	Jindo	Korea	East Asia	E	1
A11	Pungsan	Korea	East Asia	E	1
A11	Thai Ridgeback	Southeast Asia	East Asia	E	1
A11		Southeast Asia	East Asia	E	1
A11		Southeast Asia	East Asia	E	1
A12		China	East Asia	E	1
A15	Jindo	Korea	East Asia	E	1
A17	Mops	China	East Asia	E	1
A17	Mops	China	East Asia	E	1
A17		China	East Asia	E	1
A17	Akita	Japan	East Asia	E	1
A17	Hokaido	Japan	East Asia	E	1
A17	Hokaido	Japan	East Asia	E	1
A17	Hokaido	Japan	East Asia	E	1
A17	Hokaido	Japan	East Asia	E	1
A17	Kai	Japan	East Asia	E	1
A17	Kishu	Japan	East Asia	E	1
A17	Shiba	Japan	East Asia	E	1
A17	Shikoku	Japan	East Asia	E	1
A18	Chinese Crested	China	East Asia	E	1
A18	Chinese Crested	China	East Asia	E	1
A18	Chinese Crested	China	East Asia	E	1
A18		China	East Asia	E	1
A18		China	East Asia	E	1
A18	Akita	Japan	East Asia	E	1
A18	Hokaido	Japan	East Asia	E	1
A18	Iki	Japan	East Asia	E	1
A18	Iki	Japan	East Asia	E	1
A18	Kai	Japan	East Asia	E	1

Table A cont'd…

A18	Shiba	Japan	East Asia	E	1
A18	Shiba	Japan	East Asia	E	1
A18	Shiba	Japan	East Asia	E	1
A18	Shiba	Japan	East Asia	E	1
A18	Thai Ridgeback	Southeast Asia	East Asia	E	1
A18		Southeast Asia	East Asia	E	1
A19		China	East Asia	E	1
A19		China	East Asia	E	1
A19		China	East Asia	E	1
A19		China	East Asia	E	1
A19		China	East Asia	E	1
A19	Ryukyu	Japan	East Asia	E	1
A19	Ryukyu	Japan	East Asia	E	1
A19	Ryukyu	Japan	East Asia	E	1
A19	Ryukyu	Japan	East Asia	E	1
A22	Tibetan Terrier	China	East Asia	E	1
A29		China	East Asia	E	1
A29		China	East Asia	E	1
A29		China	East Asia	E	1
A29		China	East Asia	E	1
A29		China	East Asia	E	1
A29	Akita	Japan	East Asia	E	1
A29	Akita	Japan	East Asia	E	1
A29	Hokaido	Japan	East Asia	E	1
A29	Shikoku	Japan	East Asia	E	1
A29	Singing Dog	Southeast Asia	East Asia	E	1
A29		Southeast Asia	East Asia	E	1
A29		Southeast Asia	East Asia	E	1
A35	Thai Ridgeback	Southeast Asia	East Asia	E	1
A38	Chow-Chow	China	East Asia	E	1
A38		China	East Asia	E	1
A39	Shiba Inu	Japan	East Asia	E	1
A39	Shiba Inu	Japan	East Asia	E	1
A42		Southeast Asia	East Asia	E	1
A44	Tibetan Mastiff	China	East Asia	E	1
A44	Tibetan Mastiff	China	East Asia	E	1
A44		China	East Asia	E	1
A44		China	East Asia	E	1
A44		China	East Asia	E	1
A44		China	East Asia	E	1

Table A cont'd…

A44		China	East Asia	E	1
A44		Southeast Asia	East Asia	E	1
A45	Tibetan Mastiff	China	East Asia	E	1
A45	Tibetan Mastiff	China	East Asia	E	1
A46		Southeast Asia	East Asia	E	1
A47		Southeast Asia	East Asia	E	1
A50	Mops	China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A51		China	East Asia	E	1
A52		China	East Asia	E	1
A53		China	East Asia	E	1
A53		China	East Asia	E	1
A54		China	East Asia	E	1
A55		China	East Asia	E	1
A56		China	East Asia	E	3
A57		China	East Asia	E	1
A58		China	East Asia	E	1
A59		China	East Asia	E	1
A59		China	East Asia	E	1
A59		China	East Asia	E	1
A59		China	East Asia	E	1
A59		China	East Asia	E	1
A60		China	East Asia	E	1
A61		China	East Asia	E	1
A61		China	East Asia	E	1
A62		China	East Asia	E	1
A64	Shiba	Japan	East Asia	E	1
A64		Korea	East Asia	E	1
A65	Shi Tzu	China	East Asia	E	1
A67	Hokaido	Japan	East Asia	E	1

Table A cont'd…

A68	Iki	Japan	East Asia	E	1
A68	Shiba	Japan	East Asia	E	1
A68	Shiba	Japan	East Asia	E	1
A68	Shiba	Japan	East Asia	E	1
A68	Shiba	Japan	East Asia	E	1
A68	Shiba	Japan	East Asia	E	1
A68	Shiba	Japan	East Asia	E	1
A68	Shiba	Japan	East Asia	E	1
A68	Shiba	Japan	East Asia	E	1
A68	Shiba	Japan	East Asia	E	1
A68	Shiba,Shinsu	Japan	East Asia	E	1
A68	Shiba,Shinsu	Japan	East Asia	E	1
A69	Shiba	Japan	East Asia	E	1
A71	Kishu	Japan	East Asia	E	1
A72	Ryukyu	Japan	East Asia	E	1
A72	Ryukyu	Japan	East Asia	E	1
A73		China	East Asia	E	1
A73		China	East Asia	E	1
A73	Kishu	Japan	East Asia	E	1
A73	Kishu	Japan	East Asia	E	1
A73	Kishu	Japan	East Asia	E	1
A74	Shiba,Mino	Japan	East Asia	E	1
A75		Southeast Asia	East Asia	E	1
A75		Southeast Asia	East Asia	E	1
B1	Pekingese	China	East Asia	E	1
B1	Tibetan Spaniel	China	East Asia	E	1
B1	Tibetan Spaniel	China	East Asia	E	1
B1	Tibetan Spaniel	China	East Asia	E	1
B1		China	East Asia	E	1
B1		China	East Asia	E	1
B1		China	East Asia	E	1
B1		China	East Asia	E	1
B1		China	East Asia	E	1
B1		China	East Asia	E	1
B1		China	East Asia	E	1
B1		China	East Asia	E	1
B1	Kai	Japan	East Asia	E	1
B1	Ryukyu	Japan	East Asia	E	1
B1	Ryukyu	Japan	East Asia	E	1
B1	Shiba,Mino	Japan	East Asia	E	1
B1		South East Asia	East Asia	E	1
B1		South East Asia	East Asia	E	1
B2		China	East Asia	E	1

Table A cont'd…

B4	Pariah Dog	Southeast Asia	East Asia	E	1
B5	Akita	Japan	East Asia	E	1
B5	Akita Inu	Japan	East Asia	E	1
B5	Ryukyu	Japan	East Asia	E	1
B5	Pungsan	Korea	East Asia	E	1
B6	Akita	Japan	East Asia	E	1
B11	Chinese Crested	China	East Asia	E	1
B12	Kishu	Japan	East Asia	E	1
B12	Ryukyu	Japan	East Asia	E	1
B13		China	East Asia	E	1
B14	Akita	Japan	East Asia	E	1
B14	Akita	Japan	East Asia	E	1
B14	Akita	Japan	East Asia	E	1
B14	Hokaido	Japan	East Asia	E	1
B14	Hokaido	Japan	East Asia	E	1
B14	Hokaido	Japan	East Asia	E	1
B14	Iki	Japan	East Asia	E	1
B14	Iki	Japan	East Asia	E	1
B16	Shi Tzu	Japan	East Asia	E	1
B17		Mongolia	East Asia	E	1
C1		China	East Asia	E	1
C1	Ryukyu	Japan	East Asia	E	1
C1	Ryukyu	Japan	East Asia	E	1
C1	Ryukyu	Japan	East Asia	E	1
C2		China	East Asia	E	1
C2		China	East Asia	E	1
C2	Shikoku	Japan	East Asia	E	1
C3	Pekingese	China	East Asia	E	1
C3	Pekingese	China	East Asia	E	1
C3	Shiba	Japan	East Asia	E	1
C3	Shiba	Japan	East Asia	E	1
C3	Shiba	Japan	East Asia	E	1
C3	Shiba	Japan	East Asia	E	1
C3	Shiba	Japan	East Asia	E	1
C3	Shiba	Japan	East Asia	E	1
C3	Shiba	Japan	East Asia	E	1
C3	Shiba	Japan	East Asia	E	1
C3	Shiba	Japan	East Asia	E	1
C3	Shiba Inu	Japan	East Asia	E	1
C3	Shiba Inu	Japan	East Asia	E	1
C3		Southeast Asia	East Asia	E	1
C5		China	East Asia	E	1
C5		Mongolia	East Asia	E	1
C7		China	East Asia	E	1

Table A cont'd…

E1	Shiba	Japan	East Asia	E	1
E1	Pungsan	Korea	East Asia	E	1
E1		Korea	East Asia	E	1
F1	Kishu	Japan	East Asia	E	1
F3	Akita	Japan	East Asia	E	1
INDIA					
A6	Santal Hound		India	W	1
A6	Santal Hound		India	W	1
A6			India	W	1
A6			India	W	1
A6			India	W	1
A11			India	W	1
A14	Santal Hound		India	W	1
A14			India	W	1
A14			India	W	1
A14			India	W	1
A27			India	W	1
B1			India	W	1
C3			India	W	1
SIBERIA					
A1	Samoyed		Siberia	E	1
A1	Samoyed		Siberia	E	1
A2	Laika East Siberian		Siberia	E	1
A2	Laika East Siberian		Siberia	E	1
A3	Laika East Siberian		Siberia	E	1
A3	Laika East Siberian		Siberia	E	1
A5	Laika East Siberian		Siberia	E	1
A5	Laika East Siberian		Siberia	E	1
A5	Laika East Siberian		Siberia	E	1
A11	Husky Siberian		Siberia	E	1
A13	Laika West Siberian		Siberia	E	1
A18	Samoyed		Siberia	E	1
A27	Laika West Siberian		Siberia	E	1
A27	Laika West Siberian		Siberia	E	1
A29	Husky Siberian		Siberia	E	1
A29	Husky Siberian		Siberia	E	1
A41	Laika West Siberian		Siberia	E	1
B1	Samoyed		Siberia	E	1

Table A cont'd…

B1	Samoyed		Siberia	E	1
C1	Husky Siberian		Siberia	E	1
C1	Husky Siberian		Siberia	E	1
C1	Samoyed		Siberia	E	1
C4	Laika West Siberian		Siberia	E	1
F2	Husky Siberian		Siberia	E	1

APPENDIX B: Sampling Places

Table B: The details (if known) about the Turkish dog blood and buccal swap samples ,such as; sample id, name of the dog, sex, breed, parents and the address are given.

Sample ID	Dog's Name	Sex	Breed	Parents(dam x sire)	Address
Blood Samples					
K-1	Kangar	m	Kangal	Duman x Arap	SİVAS
K-2	Fıstık	f	Kangal	Altınkız x Tommy	SİVAS
K-3	Gölge	f	Kangal		SİVAS
K-4	Arak	m	Kangal	Filiz x Aral	SİVAS
Ak-5	Akbaş	m	Akbash		BURSA
K-6	Kardaş		Kangal		SİVAS
K-7	Kangalım		Kangal		SİVAS
K-8	Atılgan	m	Kangal		SİVAS
K-9	Talaş(1)	f	Kangal		SİVAS
K-10	Toraman	m	Kangal		SİVAS
K-11	Talaş(2)	m	Kangal		SİVAS
K-12	Esas	f	Kangal		SİVAS
K-13	Zalim	f	Kangal		SİVAS
K-14	Tozar	f	Kangal		SİVAS
K-15	Linda(1)	f	Kangal		SİVAS
K-16	Linda(2)	f	Kangal		SİVAS
K-17		f	Kangal		SİVAS
K-18	Lestie	f	Kangal		SİVAS
K-19	Jessie	f	Kangal		SİVAS
K-20	Kurt	f	Kangal		SİVAS
K-21	Bozo(1)	f	Kangal		SİVAS
K-22	Jimmy	f	Kangal		SİVAS
K-23	Çapar	f	Kangal		SİVAS
K-24	Çalo(1)	m	Kangal		SİVAS
K-25	Gürcey	f	Kangal		SİVAS
K-26	Bozo(2)	f	Kangal		SİVAS
K-27	Karo	m	Kangal		SİVAS

Table B cont'd…

K-28	Bulut		Kangal		SİVAS
K-29	Toro		Kangal		SİVAS
K-30	Uçar		Kangal		SİVAS
K-31	Garip		Kangal		SİVAS
K-32	Büyük Keş		Kangal		SİVAS
K-33	Taysın		Kangal		SİVAS
K-34	Çalo(2)		Kangal		SİVAS
K-35	Ejder		Kangal		SİVAS
K-36	Herkül		Kangal		SİVAS
Ak-37	Karacabey	f	Akbash		BURSA
Ak-38	Pamuk	m	Akbash		ESKİŞEHİR
Ak-39	Polar	f	Akbash		İSTANBUL
Ak-40	Akkız	f	Akbash		ESKİŞEHİR
Ak-41	Akkuş	m	Akbash		AKSARAY
Ak-42	Akbay	m	Akbash		ESKİŞEHİR
Ak-43	Beyaz	f	Akbash		ESKİŞEHİR
Ak-44	Toros	m	Akbash		BURSA
K-45	Asya	f	Kangal		SİVAS
K-46	Linda	f	Kangal		SİVAS
K-47	Karabaş	m	Kangal		SİVAS
K-48	Serkan	m	Kangal		SİVAS
K-49	Salik	f	Kangal		SİVAS
K-50	Ceylan	f	Kangal		SİVAS
K-51	Volga	m	Kangal		SİVAS
K-52	Şirin	f	Kangal		SİVAS
K-53	Dost	m	Kangal		SİVAS
K-54	Duman	m	Kangal		SİVAS
K-55	Gazal	f	Kangal		SİVAS
K-56	Dayı	m	Kangal		SİVAS
K-57	Deli	f	Kangal		SİVAS
K-58	Saro	m	Kangal		SİVAS
K-59		f	Kangal		SİVAS
K-60	Boncuk	f	Kangal		SİVAS
K-61	Badem	f	Kangal		SİVAS
K-62	Ezo	f	Kangal		SİVAS
K-63	Yavru	f	Kangal		SİVAS
Buccal Swap Samples					
K-001	Karakuyu Köpek		Kangal		SİVAS
K-002	Köpek	m	Kangal		SİVAS
K-003	Beyaz Köpek		Kangal		SİVAS
K-004	Karabaş	m	Kangal	? x Karabaş	SİVAS
K-005	Toraman	f	Kangal	Alanım x Hüsnü	SİVAS
K-006	Deliyürek	m	Kangal		SİVAS
K-007	Talan	m	Kangal	Ceylan x Duman	SİVAS
K-009	örnek no:13	m	Kangal	Çakal x Şeytan	SİVAS
K-012	Karabaş(2)	m	Kangal	Cesi x Duman	SİVAS
K-013	Herkül	f	Kangal	? x Karo	SİVAS
K-014	Karo	m	Kangal		SİVAS
K-015	Çakal	m	Kangal	Boris x Zomo	SİVAS

Table B cont'd..

K-018	Kont	m	Kangal		SİVAS
K-020	Karayaka(6)	m	Kangal	Fatoş x Zorba	SİVAS
K-023	Karabaş	f	Kangal		KAYSERİ
K-024	Zoro	m	Kangal	Pompil x Cop	SİVAS
K-026	Köpek		Kangal		SİVAS
K-028	Çapar	m	Kangal	? x Çapar	KAYSERİ
K-029	Gürcey	f	Kangal		SİVAS
K-030	Pavlo	m	Kangal		SİVAS
K-031	Aslan	m	Kangal		SİVAS
K-033	Karabaş	m	Kangal	Cesi x Duman	SİVAS
K-034	Karo	m	Kangal		SİVAS
K-036	Talaz	f	Kangal	Akrep x Heybetli	SİVAS
K-037	Kocapati(3)	m	Kangal	Cankız x Bozo	SİVAS
K-039	Herkül	m	Kangal		KAYSERİ
K-041	Karabaş	m	Kangal	Zalim x Yaman	SİVAS
K-043	Bozo	f	Kangal		SİVAS
K-044	Robin	m	Kangal	Kontes x Göğ	KAYSERİ
K-045	George	m	Kangal		SİVAS
K-046	Toraman	f	Kangal	Duman x Bozo	SİVAS
K-048	Duman	m	Kangal	Bozo x ?	SİVAS
K-049	Duman	m	Kangal	? x Karo	SİVAS
K-051	Dost	f	Kangal	Tiper x ?	SİVAS
K-052	Sultan(5)	m	Kangal		SİVAS
K-053	Talaz	m	Kangal	Mercan x Yamos	SİVAS
K-054	Küçük Tango	m	Kangal		SİVAS
K-056	Herkül	f	Kangal	Karabaş x Maço	KAYSERİ
K-059	Karabaş	f	Kangal		KAYSERİ
K-060	Kırcıl	m	Kangal	Cellat x Palak	SİVAS
K-061	Bozo	f	Kangal	Toraman x Yamus	SİVAS
K-062	Volvodo	m	Kangal	Edepsiz x Garip	KAYSERİ
K-065	Serkan	f	Kangal	Duman x Panter	SİVAS
K-068	Büyük Kız(2)	f	Kangal		SİVAS
K-069	Paşa	m	Kangal	Kontes x Göğ	KAYSERİ
K-070	Duman	f	Kangal		SİVAS
K-073	Köpek saklıva				SİVAS
K-074	Volvodo				SİVAS
K-075	Sezar	m	Kangal	Alas x Bozo	SİVAS
K-076	Keş	m	Kangal	Gürce x Duman	SİVAS
K-080	Yaman	m	Kangal	Alaç x Maço	KAYSERİ