A PRELIMINARY STUDY ON GENETIC DIFFERENTIATION AMONG TURKISH BROWN TROUT (*Salmo trutta* L.) POPULATIONS AS REVEALED BY RFLP ANALYSIS OF PCR AMPLIFIED MITOCHONDRIAL DNA SEGMENTS

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ABSTRACT

A PRELIMINARY STUDY ON GENETIC DIFFERENTIATION AMONG TURKISH BROWN TROUT (*Salmo trutta* L.) POPULATIONS AS REVEALED BY RFLP ANALYSIS OF PCR AMPLIFIED MITOCHONDRIAL DNA SEGMENTS

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In this study, based on two mtDNA regions genetic structures of brown trout (*Salmo trutta* L.) populations collected from the three distinct locations in Turkey were examined. Two regions of mitochondrial DNA corresponding to two genes: ND-1 and ND-5/6 were amplified by polymerase chain reaction and restriction fragment length polymorphism (RFLP) analyses of these two genes were carried out.

ND-1 gene was digested with the seven restriction endonucleases. A single haplotype was observed. These haplotype was fixed in all Turkish brown trout populations studied.

ND-5/6 gene was digested with the eigth restriction endonucleases. Among group variation was detected by six of the enzymes. No within group variation was observed. Two haplotypes were formed for the three populations. One of them was in the south-west Anatolian, in Alakır population. populations while the second haplotype was fixed in the north-central Anatolia, in Abant and the east Black Sea population, in Sümer.

When the results of the present study were compared with those obtained by the DNA standards, the south-west Anatolian population was found to belong the Mediteranean phylogenetic group. Similar comparisons indicated that the north-central Anatolian and the east Black Sea populations belong to the Danubian phylogenetic group.

Keywords: Brown trout populations, Polymerase chain reaction, Restriction fragment length polymorphism (RFLP), haplotype, ND-1 gene, ND 5/6 gene mtDNA variation, phylogenetic group.

ÖZ

TÜRKİYE KAHVERENGİ ALABALIK (*Salmo trutta L.*) TOPLUMLARININ GENETİK FARKLILIKLARININ PCR METODU İLE YÜKSELTGENMİŞ MİTOKONDRİYAL DNA BÖLGELERİNİN RFLP ANALİZİ İLE ORTAYA ÇIKARILMASI ÜZERİNE BİR ÖN ÇALIŞMA

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Bu çalışmada, Türkiye'nin 3 ayrı bölgesinden toplana kahverengi alabalık (*Salmo trutta* L.) toplumlarının genetik yapısı, mitokondriyal DNA nın iki böldesi açısından araştırılmıştır. Mitokondriyal DNA nın iki bölgesi: ND-1 ve ND-5/6 polimeraz zincir reaksiyonu ile çoğaltıldı ve bu genlerin keşim parçacıkları uzunluk polimorfizmi (RFLP) analizi yapıldı.

ND-1 geni 7 restriksiyon enzimi ile kesildi. Tek bir haplotip gözlendi. Bu haplotip çalışılan tüm Türkiye kahverengi alabalık toplumlarında sabit frekans göstermiştir.

ND-5/6 geni 8 restriksiyon enzimi ile kesildi. Bu enzimlerin altısı ile gruplararası varyasyon bulundu. Üç populasyonda iki haplotip gözlendi. Bu haplotiplerden birincisi güney-batı Anadolu; Alakır populasyonunda, ikincisi ise orta-kuzey Anadolu; Abant populasyonunda ve doğu Karadeniz; Sümer populasyonunda sabit frekans göstermiştir.

Bu çalışmanın sonuçları, DNA standartlarından elde edilen sonuçlarla karşılaştırıldığında günet-batı Anadolu populasyonunun Akdeniz filogenetik grubuna ait olduğu bulundu.Aynı karşılaştırma orta-kuzey Anadolu ve Doğu Karadeniz toplumlarının Tuna grubuna ait olabileceğini göstermiştir.

Anahtar kelimeler: Kahverengi alabalık toplumları, polimeraz zincir reaksiyonu, Kesim parçacıkları uzunluk polimorfizmi (RFLP), haplotip, ND-1 geni, ND-5/6 geni, mitokondriyal DNA varyasyonu, filogenetik grup.

To My Family

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ABBREVIATIONS

MtDNA: Mitochondrial DNA

NADH-1 (ND-1) gene: Nicotineamide adenine dinucleotide-1 gene

NADH-5/6 (ND-5/6) gene: Nicotineamide adenine dinucleotide -5 and 6 genes

PCR: Polymerase chain reaction

RFLP: Restriction fragment length polymorphism

MDH: Malate dehydrogenase

SOD: Superoxide dismutase

dNTP:Deoxy nucleotide tri phosphate

UPGMA: Unweighted pair-group method with arithmetic mean

EtBr: Ethidium bromide

EDTA: Ethylene diamine tetra acetic acid

TBE: Tris borate EDTA

TE: Tris EDTA

UV:Ultraviolet

OD: Optical Density

Bp: Base pair

DISPAN: Genetic Distance and Phylogenetic Analysis

CHAPTER 1

INTRODUCTION

1.1. Taxonomy and the world-wide distribution of brown trout (*Salmo trutta* L.)

The brown trout (*Salmo trutta* L.) belongs to the family Salmonidae which is usually regarded as a group of relatively primitive teleost fish due to their soft fin rays, adipose fin, and absent or incomplete oviducts. In this family, there are two subfamilies: Thymallinae (graylings) restricted to fresh-waters and Salmoniae (charr, salmon and trout) with both fresh-water and anadromous species. All members of the family breed in fresh waters. This is used as evidence to support the view that the family originated in fresh waters. Based on recent studies, rainbow trout, cut-throat trout and their close relatives have been transferred from the genus *Salmo* to the genus *Oncorhynchus*. *Salmo* is restricted to only Atlantic salmon; (*Salmo salar)* and brown trout; (*Salmo trutta*). The phylogenetic tree summarising the relationship between salmonid fishes were given in Figure1. Until the last decade, trout living in Lake Ohrid (in former Yugoslavia) and in Lake Sevan (in the Caspian Sea basin) were considered as different subspecies of

brown trout. However, the results of recent genetic studies indicated that they are populations of the same species *Salmo trutta* (Bernatchez and Osinov, 1995; Apostolidis et.al., 1996a; Osinov and Bernatchez, 1996).

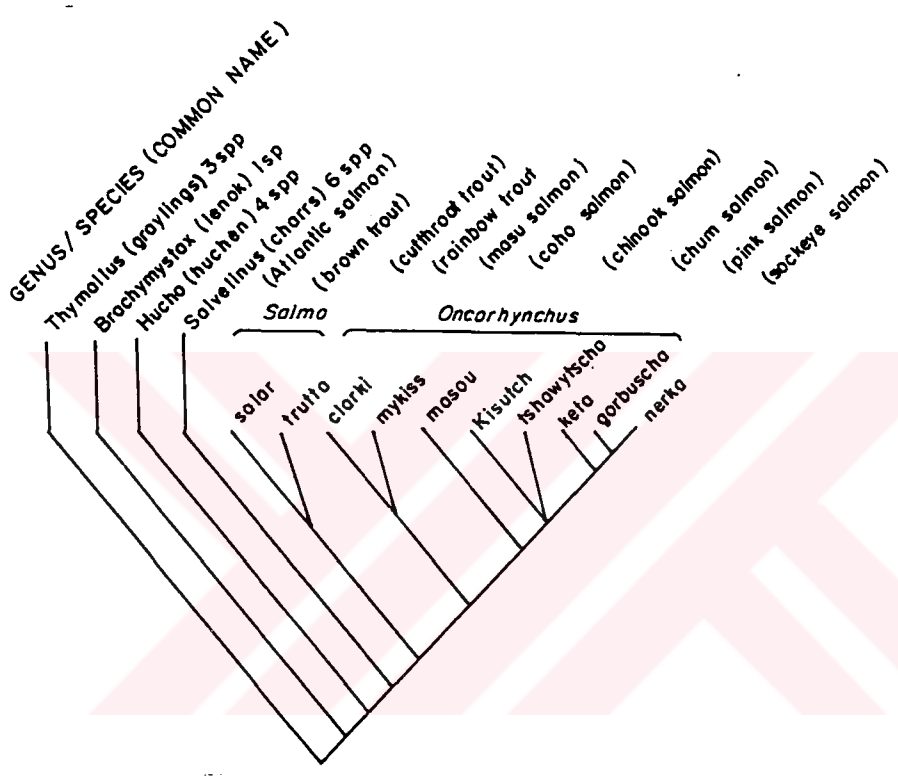


Figure1. Phylogenetic tree of relationships between salmonid fishes, based on analysis of 49 morphological characters and 24 species. Taken from Elliot, (1994)

The brown trout, *Salmo trutta* L. is a native salmonid species of Eurasia and the North Africa. Its northern borders are Iceland, the northern Scandinavia, and Russia. The western limits are simply defined by the European coastline and the southern borders by the northern coastline of Mediterranean Sea as well as the islands of Corsica, Sardinia and Sicily, and the Atlas mountains of North Africa. It is more difficult to define the eastern borders but they are probably the Ural mountains, the Caspian Sea, the Lake Aral and as far south as the upper reaches of the Orontes (Asi) River in Lebanon (Figure 2). Brown trout have been introduced successfully into at least 24 countries outside Europe. In less than 90 years the status of brown trout changed from that of a European species to that of a global species.

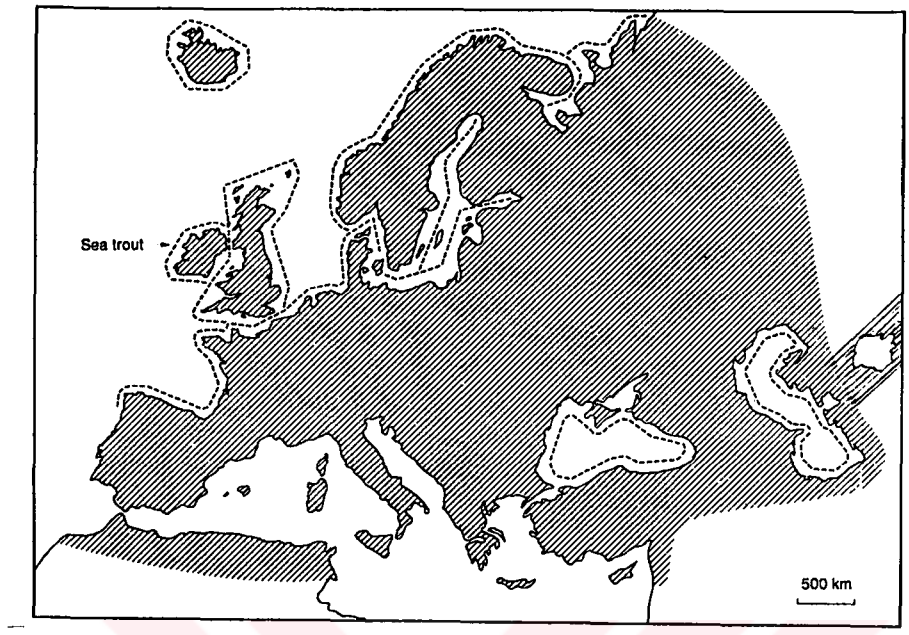


Figure 2. Native distribution of brown trout; the dashed lines indicate the distribution of sea-trout. (From Elliot, 1994)

The brown trout exhibits extreme variability and plasticity in many aspects of its morphology, ecology, and behaviour. Throughout the distribution range of the *S. trutta* populations, different forms of this species can be distinguished by their ecological and/or morphological features. Classifications based on different life patterns (anadromous, resident), habitat selection (river, lakes) and local adaptation have led to considerable taxonomic confusion which shaded the understanding of the

evolutionary history of the species. Such as, more than 50 different Linnean species have been described over the last two centuries based on variation in the species currently recognised as *Salmo trutta* (Behnke, 1986; Bernatchez et.al, 1992).

Morphological and meristic characters were also used by some authors to classify brown trout populations (Karakousis et.al, 1991). The use of morphologic criteria (body colour, pigmentation) and meristic counts (number of gill rakers, number of anal rays, etc.) to assess relationship in salmonids, are hampered by their extreme phenotypic plasticity. Thus, morphologic characters are not reliable in resolving the phylogenetic relationships in salmonids. (Bernatchez et.al., 1992; Apostolidis et.al. 1997).

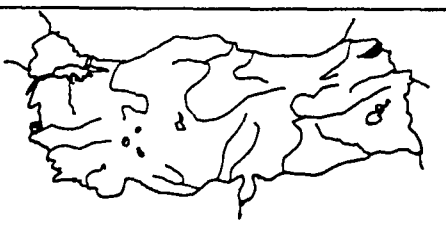
During the last three decades protein markers were used in order to assess the genetic structures of brown trout populations and their phylogenetic relationships. But recently much attention has payed to mtDNA and nuclear DNA (microsatellites, minisatellites) markers (See Section 1.3 for the explanations).

1.2. Turkish brown trout populations

Previous studies, as reviewed by Kuru (1975) were done by the employment of the morphometric and meristic characters, revealed that among the salmonid fishes *Salmo trutta* L. was the only species present in Turkey. Tortonose (1954) reviewed the trouts of Asiatic Turkey, and pointed out that there were four subspecies of *Salmo trutta.* Names of these subpecies were: *Salmo trutta macrostigma* A. DUM., which was also referred as the Mediterranean trout, *Salmo trutta abanticus* TORT., which is the trout living in Lake Abant and also accepted as an endemic subspecies, *Salmo trutta labrax* PALL. as the Pontic trout and lastly *Salmo trutta caspius* KESSL., the trout related with the one living in Caspian Sea . The distribution of these subspecies throughout Turkey was given by Geldiay and Balık (1988) (Table 1). A study by Behnke (1968), based on the results of detailed examination of three specimens that had been collected from a tributary of the Seyhan River system revealed that these specimens belong to a new species of trout, *Salmo platycephalus* (Platysalmo). Recently, platysalmo has also been recorded by Louis Bernatchez (personal communication). However the status of platysalmo remains to be verified.

Detailed studies to describe the genetic and morphological characteristics of the populations from all over the Turkey had not been done yet.

Table 1. The names and distribution of *Salmo trutta* subspecies in Turkey (From Geldiay and Balık, 1988)

NAMES OF SUBSPECIES	DISTRIBUTION SUBSPECIES
S. trutta macrostigma A. DUMERIL	
S. trutta abanticus TORTONESE	
S. trutta labrax PALLAS	
S. trutta caspius KESSL	

1.3. Use of mitochondrial DNA as a genetic marker for Salmonid populations

Early studies on the genetics of brown trout populations were based on electrophoretic analysis of protein variation. These analyses showed that brown trout is one of the most substructured species with more than 55 percent of the total genetic diversity distributed among populations in some regions (Apostolidis et.al., 1996). Recent advances in molecular biology have provided the use of new techniques in the studies of population genetics to investigate phylogenetic relationships, and to determine the degree of genetic variation both among and between brown trout populations. Various forms of molecular markers including mtDNA regions, minisatellite single locus probes, multilocus fingerprinting and microsatellites have been employed. In the present study, mtDNA regions were used as markers. The use of DNA markers has some advantages over the protein markers. Tissue collection and storage are easy in DNA studies since no specific temperature is required as in protein studies. In order to obtain reliable results, tissues collected for protein studies should be kept in at least -20 C^0 from the time of their collection until their analysis. For the DNA studies tissues can be preserved in 90% ethanol for several years in room temperature. Only single and small amount of tissue is required in

DNA studies. In protein studies different tissues are required to study different enzyme systems. Furthermore, in DNA studies of salmonids there is no need to kill fishes since adipose fin can be readily used to extract DNA and adipose fin can be cut without causing the death of the fish. MtDNA studies provided some additional advantages. Since mtDNA is inherited maternally it has one quarter effective population size in comparison to nuclear DNA. A smaller effective population size means that genetic drift can cause frequency differences between isolated gene pools more readily in mtDNA than in nuclear genes. In many organisms, the mtDNA also seems to accumulate mutations more rapidly than nuclear genes due to lack of DNA repair mechanism. In other words, mtDNA holds greater genetic variability. Moreover it is more sensitive to genetic drift therefore it is more likely to show differences between populations or species (Carvalho and Pitcher, 1995; Ferguson et.al.,1995). The maternal mode of inheritance of mtDNA makes it a valuable genealogical tool for tracing the history of female lineages even after interbreeding and thus nuclear introgression has taken place (Hynes et.al., 1989). Use of mtDNA primers and polymerase chain reaction amplification of selected regions have made examination of mtDNA variation considerably faster and easier. All of these advantages make mtDNA attractive in both the studies of systematics and studies of population. Although there are several advantages it also has some drawbacks. The methods used in mitochondrial

DNA analysis are expensive as well as more difficult to apply. Unfortunately, sometimes little information can be gained at the end of mtDNA studies. In order to characterise a population genetically mtDNA alone may not give a full information. Thus some other markers should be included as well (Bernatchez et.al., 1992; Carvalho and Pitcher, 1995; Ferguson et.al., 1995).

In recent studies, the analysis of mtDNA sequence variation among brown trout populations from various locations have revealed the existence of six major geographically disjunct phylogenetic groups (Bernatchez et.al., 1992; Giuffra et.al., 1994; Bernatchez and Osinov, 1995; Osinov and Bernatchez, 1996; Apostolidis et.al., 1997; Garcia-Marin et.al., 1998). These groups are namely the North-Atlantic, the South-Atlantic, the Mediterranean, the Po, the Adriatic and the Danube (Figure 3.) It was previously claimed that the whole Mediterranean basin, from Spain to Turkey belonged to one of the three major phylogenetic groupings beside Atlantic and Danube groups (Bernatchez and Osinov, 1995). The present study, based on polymerase chain reaction dependent restriction fragment length polymorphism (PCR-RFLP) in mtDNA, will attempt to identify the place of Turkey among these phylogenetic groups.

from the nuclear DNA. MtDNA is 16 000 to 20 000 base pair in size and it is circular in shape. It contains over 30 genes and since no recombination is seen, it is treated as a single locus (Figure 4) (Carvalho and Pitcher, 1995).

In this study these ND genes were used as markers since they are widely used to detect both among and within group variations in brown trout populations (Cronin et.al., 1993; Hall and Nawrocki, 1995; Hynes et.al., 1996). ND-1 gene is about 2000 base pairs in size while that of ND-5/6 gene is approximately 2500 base pairs long (Cronin et.al., 1993).

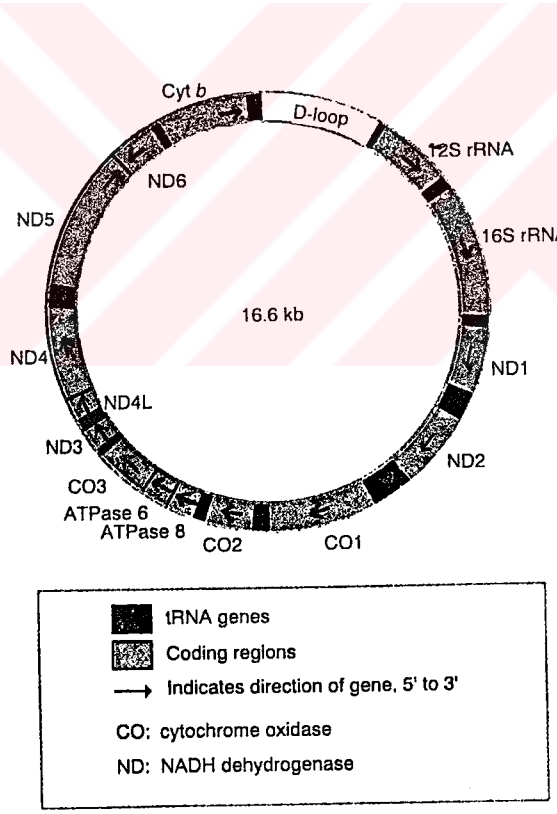


Figure 4. The mitochondrial map (From Lewin, 1997)

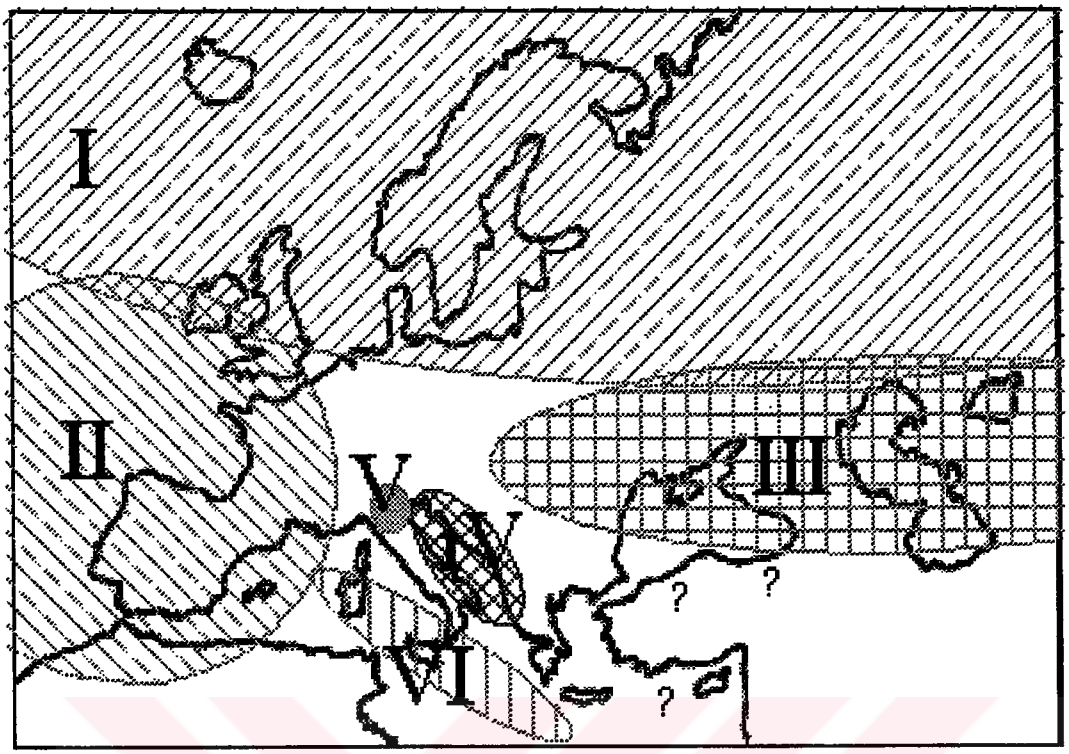


Figure 3. Distribution of phylogenetic groups of brown trout

I) North Atlantic, II) South Atlantic, III) Danube IV) Adriatic V) Po VI) Mediterranean

1.4.Genetic markers used in this study

One of the most oftenly studied DNA in animals is the mitochondrial DNA for several reasons which were explained in the Section 1.3. Mitochondria has its own DNA which is physically separate

from the nuclear DNA. MtDNA is 16 000 to 20 000 base pair in size and it is circular in shape. It contains over 30 genes and since no recombination is seen, it is treated as a single locus (Figure 4) (Carvalho and Pitcher, 1995).

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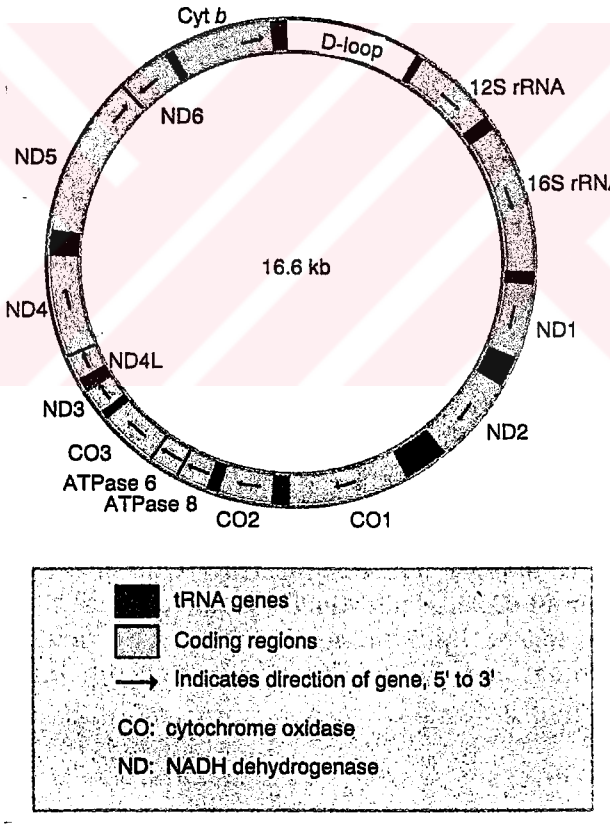

Figure 4. The mitochondrial map (From Lewin, 1997)

1.5.The aim of the study

The aim of the present study is;

1- To establish the methods for studying restriction fragment length polymorphism (RFLP) in mtDNA genes after the amplification by polymerase chain reaction,

2- To determine the genetic structures of Turkish populations by using mtDNA

PCR-RFLP approach, based on ND-1 and ND-5/6 genes,

3- To find out genetic variability levels within the populations,

4- To find out genetic variability between the populations

5- To compare the results of the present study with those of the results obtained from the populations from other countries.

6- To find out which phylogenetic group(s) do Turkish populations belong,

Finally, in addition to the present study a parallel study based on protein polymorphism has just been finished, and there was a previous comparative study based on morphology data, the level of congruence between the three different data sets (mtDNA, protein, morphology) will be discussed.

CHAPTER 2

MATERIALS AND METHODS

In this chapter materials, methods and statistical analyses of the present study will be presented.

In the present study brown trout samples collected from three different localities were analysed using mtDNA PCR-RFLP approach.

2.1. The study areas

The samples used in this study were collected between 1997 and 1998. The geographical locations of the sites are shown on the map in the Figure 5. Four populations namely Alakır1, Alakır2, Alakır3 and Eşen were collected from Antalya, in south-west of Anatolia, two populations were collected from Lake Abant in the north–central Anatolia in different times ,

finally Fırtına and Sümer populations were collected around Rize, in east Black Sea.

The sample sizes from the each population were as follow; Alakır1 (15), Alakır2 (10), Alakır3 (10), Eşen (20), Abant1 (16), Abant2 (16), Fırtına (20) and Sümer (20). The Alakır1, Alakır2 and Alakır3 populations were collected from the different parts of Alakır stream. Eşen population was collected from Eşen stream. Abant1 and Abant2 populations were collected from different parts of Lake Abant at different times. Fırtına population was collected from Fırtına River. Sümer population was collected from Sümer River. Both of Fırtına and Sümer rivers flow into Black Sea. The samples from these rivers were collected about 3- 4 kilometer away from the seashore. Totally 127 individuals were collected from these localities.

2.2. Sampling

The samples from Alakır and Eşen were collected by electrofishing while the samples from Lake Abant were collected both by gill netting and angling, and the samples around Rize were collected by gill netting and electrofishing. In rivers, in order to avoid capturing members of the same

family sampling sites were chosen such that they were at least 500 metre apart.

2.3. Sample preparation

After samples were transported to laboratory fishes were dissected and approximately 200-500 miligram of the liver and muscle tissues were placed into 90 % ethanol. Then tissues were stored in –20 ^{0}C until the DNA extraction time.

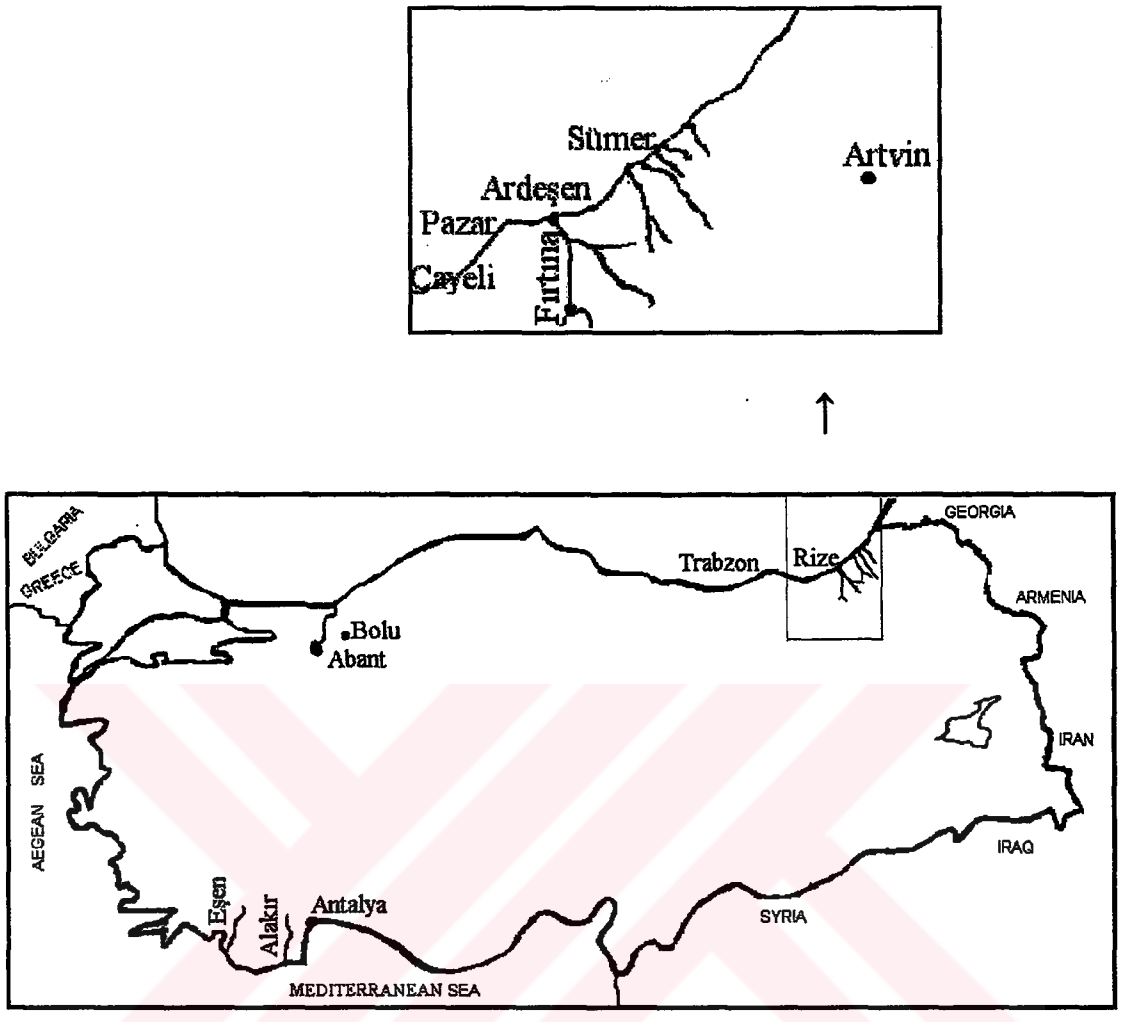

Figure 5. Collection sites of the samples

2.4. DNA Analysis

In this part detailed information will be given about the experimental methods that are used in DNA analysis.

2.4.1. Isolation of total genomic DNA

For the DNA analysis total nucleic acids were isolated from tissues in accordance with Taggart et.al. (1992). Hundred mg skeletal muscle or 50 mg liver was placed in 1.5-1.75 microcentrifuge tube with 375 µl 0.2 M EDTA- sarcosyl (0.5 %) solution. Tissue was chopped with a sterilised scissor by wearing gloves. Twenty five µl proteinase-K [20 mg /ml (50 mM pH:8.0 Tris solution)] was added onto chopped tissue. This tissue was incubated 15 hours at 37^0C . Fifteen µl DNAse free RNAse (2mg/ml) (kept in 100^0C waterbath for 20 minutes) was added to the same tube. This was followed by a 1.5 hours incubation period at 37^0C. After incubation period 400 µl phenol (pH 8.0) was added into the same tube. Tube was shaken vigorously by hand for 20 second. Then 1 hour gentle mixing was carried out. The same amount, 400 µl chloroform: isoamyl alcohol (24:1) was added to the tube and this mixture was shaken vigorously for 20 seconds by

hand. Then 30 minute gentle mixing was followed.. After gentle mixing tube was spinned in microcentrifuge at 12000 g for 15 minutes. Upper aqueous layer was transferred with a cut pipet tip to another sterilised microcentrifuge tube. Three volumes of 88% cold ethanol from 4^0C was added to the removed aqueous layer and tube was mixed abruptly and rapidly 5-6 times. When DNA was precipitated ethanol was carefully removed from tube. One mililiter of 70% ice cold ethanol (from -20^0C) was added into DNA pellet and precipitated DNA was left in ethanol for overnight (15 hours). After 15 hours, ethanol was removed by using vacuum dryer. Dried DNA pellet was dissolved in 100 µl 1X TE buffer (10mM Tris, 1mM EDTA pH:8.0) and stored in 4^0C.

2.4.1.1. Observation of total DNA bands

The presence of isolated DNA was checked 0.8% agarose gel. Agarose gels were prepared by mixing 0.24 gram agarose with 30 ml 0.5 X TBE buffer (54 g Tris, 27.5 g boric acid, 4.65 g $Na_2EDTA2H_2O$ into 1 litre

ddH_2O pH:8.0). Agarose in this mixture was dissolved in microwave oven. To this mixture 1.0 µl Ethidium Bromide (EtBr) (10mg/ml) was added. Then mixture was poured into gel tray (which has a comb to make the wells for the application of the DNA containing samples) and allowed to polymerise. Comb was removed from gel without disrupting it. To visualise the front of the moving mixture on the gel during electrophoresis, 1µl DNA sample was mixed with 5µl Bromophenol blue solution [4g sucrose and 2.5 mg bromophenol blue mixed with 6 ml 1XTE buffer (equilibrated with HCl pH:8.0) and then added up to 10 ml with TE buffer] and 8 µl distilled sterile water. All of the 15µl mixture is applied to wells. Gel was run at 100 V voltage and 30 A current for 30 minutes (until bromophenol blue was 2-3 centimeter away from wells) in 0.5X TBE tank buffer.. Then the gel was visualized under UV light. DNA bands seems as wide sharp bands without any other bands behind or in front of them (Figure 6).

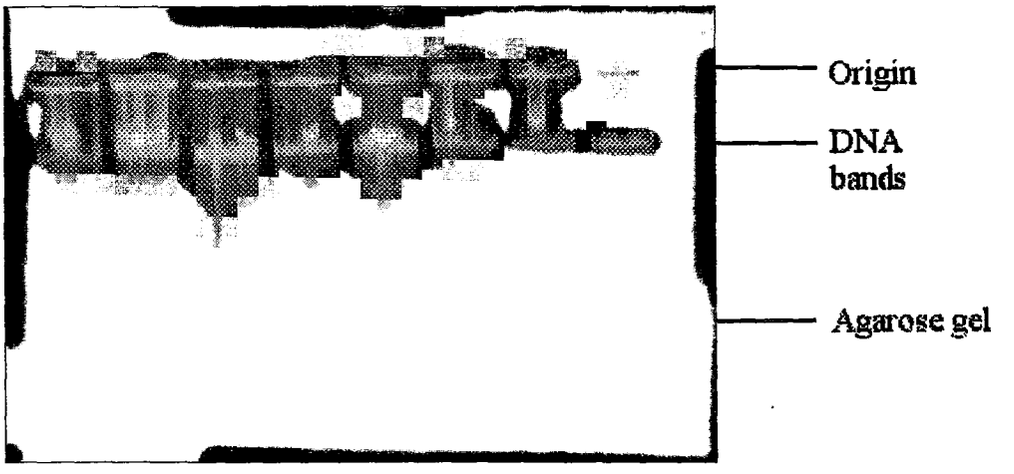

Figure 6. Total DNA bands on agarose gel

2.4.2.Polymerase chain reaction (PCR)

ND-1 and ND-5/6 regions of mitochondrial DNA of brown trouts were amplified by (PCR). Components of the PCR mixtures for ND-1 and ND-5/6 regions, sequences of primer pairs, the conditions of the PCR reactions and the number of individuals studied from each population for each gene were summarized in Table 2., Table 3. Table 4 and Table5., respectively.

Table 2. Components of the PCR mixture for ND-1 and ND-5/6 regions

Chemical	Amount for ND-1 Region	Amount for ND-5/6 Region
10X TBE Reaction buffer (MBI Fermentas)	5.0 µl (1X)	5.0 µl (1X)
25mM $MgCl_2$	3.0 µl (1.5 mM)	5.0 µl (2.5mM)
5mM dNTPs	2.0 µl (200 µM)	2.0 µl (200 µM)
Primer 1	1.0 µl (100 ng)	1.0 µl (100 ng)
Primer 2	1.0 µl (100 ng)	1.0 µl (100 ng)
DNA template	3-5 µl (100 ng)	3-5 µl (100 ng)
Taq Polymerase (5u/µl)	0.5 µl (2.5 U)	0.5 µl (2.5 U)
Distilled water	34.5 – 32.5 µl	32.5 – 30.5 µl
Total	50 µl	50 µl

Table 3. Sequences of the primer pairs for ND-1 and ND-5/6 regions

Region	Primer Name	Primer Sequence
ND-1	RHND1 Forward	5′-TGC CCC CAT GGA AGA GAT TAT-3′
ND-1	RHND1 Reverse	5′-AGT GGG AGC TGG CAA AGG TGA -3′
ND-5/6	NAD-5F:	5'-CCA CAC CCG AGA ACA CCT ACT TAT -3'
ND-5/6	NAD-6R	5'-GAG GTC GAC TAG TGC GTC ATT AGC -3'

Table 4. Conditions of the cycles during the PCR amplifications of the ND-1 and ND-5/6 regions

Temperature (C^0)	Time	Number of cycle(s)
95 C^0	5min	1 cycle
95 C^0	1min 30 sec	30 cycles
63 C^0	1min 30 sec	
72 C^0	2 min 30 sec	
72 C^0	10 min	1 cycle

Numbers of amplification cycles are the same for ND-1 and ND-5/6 regions.

In every PCR reactions negative control (10XTBE, $MgCl_2$, dNTPs, primer1, primer2, taq polymerase and distilled water was used in same amounts that are given in the Table2.) was carried out in order to check if there was any contamination in chemicals used in PCR mixture. PCR mixture in control tube contained everything except the DNA sample.

Optical density (OD) of each DNA sample was measured prior to PCR amplification in order to decide DNA amount that will be used in polymerase chain reaction. Required amounts were changed between 100ng to 500ng from sample to sample, in this study.

Table 5. The number of individuals studied for ND-1 and ND-5/6 genes

Sampling Localities	ND-1	ND-5/6
Alakır (Alakır1,Alakır2, Alakır3)	35	10
Eşen	20	-
Abant (Abant1, Abant2)	32	32
Sümer	20	10
Fırtına	20	-
Total	127	52

2.4.2.1. Observation of PCR bands

To visualise PCR products 0.8 % agarose gel was used. In the mixture 0.32 grams agarose was mixed with 40 ml 0.5XTBE buffer and melted in microwave oven. To the gel 1.0 µl Ethidium Bromide (EtBr) (10mg/ml) was added, then 10 µl PCR product was mixed with 5 µl bromophenol blue and loaded into the wells. Gel was run for 30 minutes in 0.5XTBE tank buffer. PCR bands were visualised under UV illuminator and photographed by Polaroid camera (Fig 7/A). As a molecular weigth size standard a λ/*HindIII* digest ladder (MBI Fermentas) was used. Lambda marker had seven bands which were 23130 kb, 9416 kb, 6557 kb, 4361 kb, 2322 kb, 2027 kb, and 564 kb in size

2.4.3. Digestion of amplified PCR products with restriction endonucleases

Amplified ND-1 and ND-5/6 regions were digested with 7 and 8 restriction endonucleases respectively. Amplified ND-1 region was digested with *AluI, AvaII, EcoRI, HaeIII, HinfI, HpaII, XbaI*. In addition to

these endonucleases ND-5/6 region was also digested with *TaqI* enzyme. Some of these enzymes are hexameric while others are multipentameric or tetrameric. Restriction enzymes used in this study were given in Table 7 along with restriction recognition sites, optimum temperatures at which they work and their trademarks.

The patterns (morphs) obtained after the each digestion were labelled with a capital letter. If the observed morph was seen previously, the same letter given by him/her was used. Otherwise, a new letter different than the already used ones was used.

Restriction digests were set up in a volume of approximately 14 µl per sample, consisting of 5-8 µl PCR product, 1.4µl MBI Fermentas's 10X Reaction buffer for *AluI, AvaII, EcoRI, HaeIII, HpaII, TaqI and XbaI* digestions or 2.8 µl MBI Fermentas's 10X Reaction buffer for *HinfI* digestion, 7.6 µl distilled water for *AluI, AvaII, EcoRI, HaeIII, HpaII, TaqI and XbaI* digestions or 6.2 µl distilled water for *HinfI* digestion and 2.5 units MBI Fermentas's restriction enzyme. The digests were incubated for overnight (15 hours) at 37^0C. The optimum temperature and digestion period were different for *TaqI* digests. They were overlaid with mineral oil and incubated at 65^0C for 5 hours.

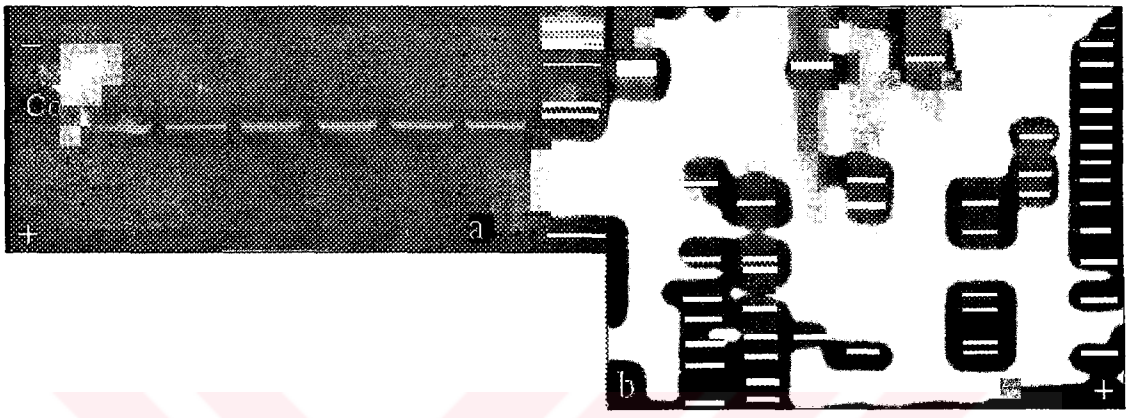

Figure 7. a) PCR products, negative control "Co" in the first lane and λ marker/ladder in the last lane

b) An example for restriction bands after cutting with *AvaII*, *AluI*, *XbaI*, *HpaII*, *EcoRI*, *HaeIII* and *HinfI* enzymes (from lef-t to right), undigested PCR product is in the first lane and 100 bp ladder is in the last lane

Table 6. Restriction endonucleases used in this study

Restriction enzyme	Recognised sequence	Optimum temperature	Company
AluI (Tetrameric)	5'-AG ↓ CT-3' 3'-TC ↑ GA-5'	37°C	MBI Fermentas
AvaII (Multipentameric)	T 5'-G↓GAC C-3' 3'-C CTG↑G-5' A	37°C	MBI Fermentas
EcoRI (Hexameric)	5'-G↓AATT C-3' 3'-C TTAA↑G-5'	37°C	MBI Fermentas
HaeIII (Tetrameric)	5'-GG ↓ CC -3' 3'-CC ↑ GG -5'	37°C	MBI Fermentas
HinfI (Multipentameric)	5'-G↓ ANT C-3' 3'-C TNA ↑G-5' where N: G, A, T or C.	37°C	MBI Fermentas
HpaII (Tetrameric)	5'-C↓ CG G-3' 3'-G GC↑C-5'	37°C	MBI Fermentas
TaqI (Tetrameric)	5'-T↓GC A-3' 3'-A CG↑T-5'	65°C	Sigma
XbaI (Hexameric)	5'-T ↓CTAG A-3' 3'-A GATC↑T-5'	37°C	MBI Fermentas

2.4.3.1. Observation of RFLP bands

After incubation period digested samples were electrophoresed in 2 – 3 % agarose gels (1 g agarose mixed with 50 ml 0.5XTBE buffer) at 100V, 30 mA for 1-1 ½ hours. The tank buffer was 0.5X TBE was used. MBI Fermentas's 100 bp plus ladder was used as a molecular size standard. The fragments were visualised under UV light, and recorded on Polaroid film (Fig 7/B). Each restriction pattern of amplified segment was labelled by a capital letter. Haplotypes (composite genotypes) were designated by 15 letters i.e. one letter for each of the restriction enzymes used for screening first for ND-1 region then followed by corresponding letter for ND-5/6 region.

2.5. DNA standards

Mediteranean and Atlantic DNA standards provided by Rene Guyomard (Laboratoire de Genetique des poissons, INRA, France) were PCR amplified and the amplified ND-1 and ND-5/6 regions were digested with the same enzymes that are used in this study. The results obtained as a result of digestion of amplified ND-1 and ND-5/6 regions were compared with that of all samples analysed in this study.

2.6. Statistical analysis employed for mtDNA data

In this study, as a measure of genetic variation, proportion of nucleotide difference ($\hat{p}$) between the populations were calculated (Nei, M., 1987).

$$\hat{p} = \hat{p}_1 \cdot \frac{\sum_i r_i \cdot (\hat{m}_i - m_{XY\,i})}{\frac{\left[\left\{1-(1-\hat{p}_1)^{r_i}\right\}\cdot\left\{2-(1-\hat{p}_1)^{r_i}\right\}\right]}{\sum_i r_i \cdot \hat{m}_i}} \qquad (2.1)$$
$$\left[2-(1-\hat{p}_1)^{r_i}\right]$$

Explanation of the symbols were as follows:

$\hat{p}$: The proportion of nucleotide differences is given by

$$\hat{p} = 1 - \left(\frac{m_{XY}}{\frac{m_X + m_Y}{2}} \right)^{\frac{1}{r}} \qquad (2.2)$$

r : The number of nucleotides in the recognition sequence of a restriction enzyme, For example $r = 4$ for tetrameric restriction enzymes.

m_X : The numbers of restriction sites for DNA sequence X

m_Y : The numbers of restriction sites for DNA sequence Y,

m_{XY} : The numbers of restriction sites shared by the two sequences,

$$\hat{m}_i = \frac{m_X + m_Y}{2}$$

$\hat{p}_1$ is the $\hat{p}$ value obtained in the equation (2.2.)

In each iteration newly calculated $\hat{p}$ value will be used instead of $\hat{p}_1$ and iteration will be carried out until a constant $\hat{p}$ value is reached..

Haplotype diversity and its variance was calculated using the formulae given in Nei, M., (1987). These formulae are given below.

$$h = \frac{2 \cdot n \cdot \left(1 - \sum x_i^2\right)}{2 \cdot n - 1} \tag{2.3}$$

where,

n is the sample size and the x_i^2 is the squared haplotype frequencies. The variance of this heterozygosity is given by

$$V(h) = \frac{2}{2 \cdot n(2 \cdot n - 1)} \cdot \left\{ [2(2 \cdot n - 2)] \cdot \left[\sum x_i^3 - \left(\sum x_i^2 \right)^2 \right] + \sum x_i^2 - \left(\sum x_i^2 \right)^2 \right\} \tag{2.4}$$

and the standard error would be the square root of $V(h)$.

UPGMA (un-weighted pair-group method with arithmetic mean) dendogram was also constructed by using the package program NTSYS (Rohlf, 1997).

G_{ST} (gene differentiation among subpopulations), H_T (gene diversity in total population) and H_S (average gene/haplotype diversity within subpopulations), were calculated by using package program DISPAN (Ota, 1993).

CHAPTER 3

RESULTS

In this chapter results observed in native brown trout populations of Turkey will be presented. To be able to identify the phylogenetic groups of brown trout presented in Turkey the results of two standards corresponding to the Atlantic and Mediterranean stocks will also be compared the results of present study.

3.1. Mitochondrial DNA

In this study brown trout populations from the 3 localities were screened for two regions of mt-DNA. First of these regions is ND-1 region and the second region is ND-5/6. The results concerning these regions are given below.

3.1.1. ND-1 Region of mitochondrial DNA

The restriction fragments obtained as a result of *AluI*, *AvaII*, *EcoRI*, *HaeIII*, *HinfI*, *HpaII* and *XbaI* digestions of PCR amplified ND-1 genes are given below..

When *AluI* enzyme was applied on the amplified ND-1 region, the eight fragments with the approximate sizes 620 bp, 410 bp, 410 bp, 270 bp, 200 bp, 180 bp, 140 bp, 120 bp were observed. All the populations studied showed the same pattern (Figure 8.). This pattern showed similarities with the morph A (660 bp, 390 bp, 290 bp, 230 bp, 200 bp, 200 bp) which was recorded by Hansen and Loeschcke (1996). *AluI* morph obtained in this study was labelled with letter ' D' since previously this morph was not observed by someone else before.

AvaII digestion of the amplified ND-1 region yielded the nine different fragments (approximate sizes of the fragments were 730 bp, 410 bp, 290 bp, 240 bp, 190 bp, 190 bp, 160 bp, 120 bp, 100 bp). All populations studied showed the same pattern (Figure 8.). This pattern was similar to the morph B (750 bp, 410 bp, 290 bp, 210 bp, 170 bp, 150 bp) which was observed by Hansen and Loeschcke (1996). *AvaII* morph

obtained in this study was labelled with letter ' C' because of the same reason mentioned above.

EcoRI digestion of the amplified ND-1 region gave rise to only one fragment which has an approximate size of 2450 bp. In the all populations studied same morph was observed (Figure 8). This morph was labelled with letter 'A' since no information is available in the literature about the restriction fragments obtained by the application of *EcoRI* on ND-1 region.

HaeIII digestion yielded 6 fragments (approximate sizes of the fragments were 620 bp, 480 bp, 290bp, 250bp, 200bp, 180bp) All the populations analysed showed the same pattern (Figure 8). This pattern was similar to morph C (660 bp, 500 bp, 280 bp, 210 bp, 200 bp, 110 bp) which was observed in the populations studied by Hansen and Loeschcke (1996). *HaeIII* morph obtained in this study was labelled with letter 'D' since none of the morphs A, B and C, which were recorded by the other authors, were identical to the fragments that were obtained in the present study.

HinfI digestion produced the three fragments (approximate sizes of fragments were 1050 bp, 790 bp, 640 bp). All the populations studied showed the same pattern (Figure 8). This pattern showed similarities with morph A (1000 bp, 540 bp, 500 bp) which was seen in the populations studied by Hansen and Loeschcke, (1996). *HinfI* morph obtained in this

study was labelled with a letter of 'C' because the morphs A and B observed by other authors were not the same with the morph that we obtained.

HpaII digestion of the amplified ND-1 region yielded to the four fragments (approximate sizes of the fragments were 790 bp, 615 bp, 615 bp, 190 bp,). All the populations studied showed the same pattern (Figure 8). This pattern was similar to morph B (720 bp, 520 bp, 460 bp, 180 bp, 130 bp) which was observed in the study conducted by Hansen and Loeschcke (1996). The morph observed in this study was labelled with letter 'C' because of the same reason mentioned above.

XbaI digestion of the amplified ND-1 region produced the two fragments (approximate sizes of the fragments were 2200 bp and 250 bp). All the populations analysed showed the same pattern (Figure 8). This pattern was not defined in literature. It is labeled with letter "A".

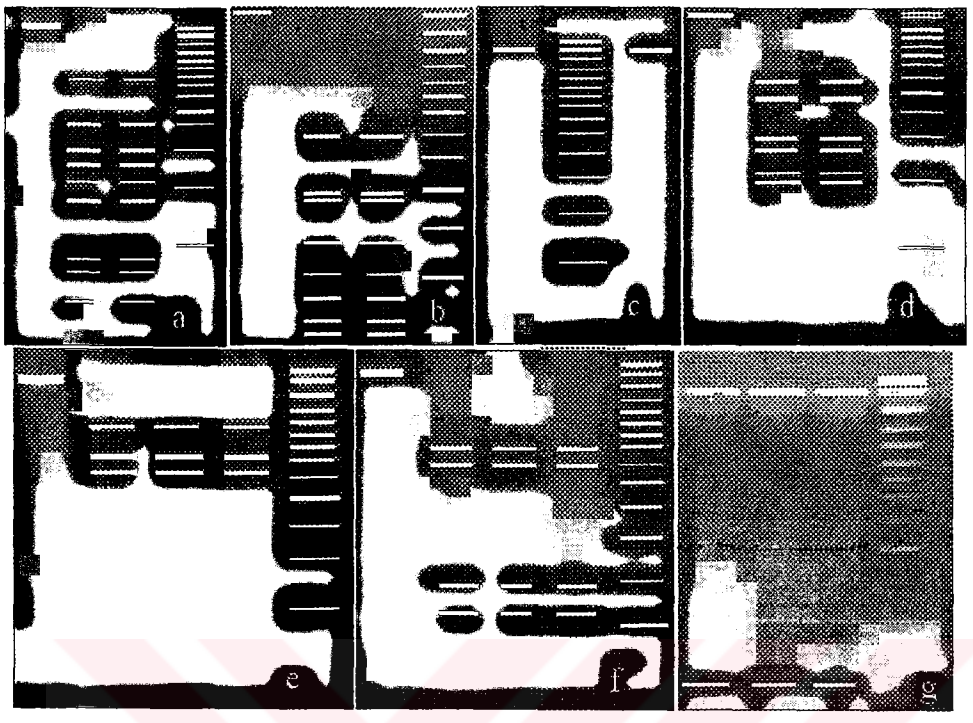

Figure 8. Restriction bands of ND-1 region after digesting with a) *AvaII*, b) *AluI*, c) *EcoRI*, d) *HaeIII*, e) *HinfI*, f) *HpaII*, g) *XbaI*

3.1.1.1. Mediterranean and Atlantic DNA standards

Two DNA standards which were known to belong to the Mediteranean and Atlantic stocks, were amplified with ND-1 primers. PCR products of ND-1 region of these samples were digested with 7 restriction endonucleases (*AluI, AvaII, EcoRI, HaeIII, HinfI, HpaII* and *XbaI)* in order

to observe if there were any genetic differentiation between Mediterranean and Atlantic stocks in terms of ND-1 region. It was seen that both stocks showed the same morphs when digested with the same restriction endonucleases. Hence, it was seen that ND-1 region could not differentiate Mediterranean and Atlantic stocks.

3.1.2. NADH-5/6 (ND-5/6) Region of mitochondrial DNA

The restriction fragments obtained as a result of *AluI, AvaII, EcoRI, HaeIII, HinfI, HpaII, XbaI, TaqI* digestions of PCR amplified ND-5/6 regions are given below. The same labelling method used for ND-1 region was used for ND-5/6 region.

For six of the restriction endonucleases genetic variation between northern (Sümer and Abant) and Alakır populations was found. These enzymes are *AluI, AvaII, HaeIII, HpaII, XbaI* and *TaqI*. With *EcoRI* and *HinfI* no genetic variation was observed.

AvaII digestion yielded 2 fragments (approximate sizes of fragments were 1640 bp and 1130 bp) in the Sümer and Abant populations, while 3 fragments (approximate sizes of fragments were 1640 bp, 640 bp, 530 bp) were obtained in the Alakır populations (Figure 9. and Figure 10).

In Alakır populations one restriction site was gained or one restriction site was lost in Sümer and Abant populations.

Two morphs obtained as a result of *AvaII* digestion were similar to morphs defined as morph C (1700 bp, 810 bp) and morph B (1700 bp, 570 bp, 240 bp)) by Apostolidis et.al. (1996b). Hansen and Loeschcke (1996) defined similar morphs as morph A (1740 bp, 840 bp) and morph B (1740 bp, 560 bp, 240 bp

Morph obtained in the Sümer and Abant populations was labelled with letter 'D' while morph seen in the Alakır populations was labelled with letter 'E'.

HpaII digestion gave rise to 4 fragments (approximate sizes of fragments were 1200 bp, 1100 bp, 340 bp, 215 bp) in the Sümer and Abant populations while 5 fragments (approximate sizes of fragments 1100 bp, 620 bp, 510 bp, 340 bp, 250 bp) were obtained in the Alakır populations (Figure 9 and Figure 10.). In the Alakır populations one restriction site was gained or one restriction site was lost in the Sümer and Abant populations.

As a result of *HpaII* digestion two different morphs were obtained. These morphs were similar to morph B (1090 bp, 760 bp, 320 bp, 290 bp,) and morph A (760 bp, 570 bp, 520 bp, 320 bp, 290 bp) which were defined by Apostolidis et.al.1996). Morph obtained in Sümer and Abant

populations was labelled with letter 'E' while the morph observed in Alakır populations was labelled with letter 'F'

HaeIII digestion yielded 6 fragments (approximate sizes of fragments were 930 bp, 880 bp, 760 bp, 125 bp, 90 bp, 70 bp) in the Sümer and Abant populations while 5 fragments (approximate sizes of fragments were 930 bp, 885 bp, 760 bp, 170 bp, 130 bp) in the Alakır populations (Figure 9. and Figure 10.). In Alakır populations one restriction site was lost or one restriction site was gained in Sümer and Abant populations.

HaeII morphs which were similar to morph A (870 bp, 710 bp, 570 bp, 170 bp, 130 bp) and morph C (870 bp, 710 bp, 570 bp, 190 bp,170 bp) defined by Apostolidis et.al., (1996)

The morphs observed in Sümer and Abant, and Alakır populations were labelled with letters 'D' and 'E', respectively.

AluI digestion gave rise to 8 fragments (approximate sizes of fragments were 760 bp, 440 bp, 280 bp, 250 bp, 220 bp, 190 bp, 140 bp and 100 bp) in the Sümer and Abant populations while 7 fragments (approximate sizes of fragments were 1070 bp, 470 bp, 330 bp, 290 bp, 270 bp, 250 bp, 180 bp) in the Alakır populations (Figure 9 and Figure 10.). These observations indicated that one restriction site was lost in Alakır

populations or one restriction site was gained in Sümer and Abant populations.

Apostolidis et.al., (1996) labeled similar morphs as morph A (940 bp, 490 bp, 280 bp, 250 bp, 250 bp, 230 bp, 150 bp, 100 bp, 20 bp) and morph B (1090 bp, 490 bp, 280 bp, 250 bp, 230 bp, 100 bp, 20 bp)

AluI morph obtained in the Sümer and Abant populations was labelled as 'G' while morph seen in the Alakır populations labelled with letter 'H'.

XbaI digestion produced 2 fragments (approximate sizes of fragments were 1720 bp and 550 bp) in the Sümer and Abant populations and 1fragment (approximate size of fragment was 2500 bp) in the Alakır population (Figure 9. and Figure 10.). In the Alakır population one restriction site was lost or in the Sümer and Abant populations one restriction site was gained.

XbaI digestion gave rise to two morphs. Similar morphs were observed by Hansen and Loeschcke (1996) and these morphs were defined as morph A (2570 bp) and morph B(2320 bp, 250 bp). Morphs obtained in the present study were labelled with 'C' and 'D' respectively for Sümer and Abant, and Alakır populations.

TaqI digestion produced 5 fragments (approximate size of fragments were 710 bp, 560 bp, 480 bp, 250 bp, 190 bp) in the Sümer and Abant populations, in Alakır populations another 5 fragments different than those of the Sümer and Abant populations (approximate sizes of fragments were 800 bp, 690 bp, 550 bp, 470 bp, 260 bp) were observed (Figure 9 and Figure 10.). These morps were similar to morph A (780 bp, 580 bp, 500 bp, 310 bp, 290 bp, 230 bp) obtained by Apostolidis et.al., (1996). These morphs were labeled with letter 'D' for the Sümer and Abant populations and with letter 'E' for the Alakır populations.

Digestion with *EcoRI* yielded 2 fragments (approximate sizes of fragments were 1870 bp, 580 bp) in the all populations studied (Figure 9. and Figure 10.). The morph obtained in this study was labelled with letter 'B'. This enzyme could not differentiate populations from each other (Apostolidis et.al.,1996).

HinfI digestion yielded 7 fragments (approximate sizes of fragments were 650 bp, 550 bp, 420 bp, 300 bp, 270 bp, 230 bp, 190 bp) in all populations studied (Figure 9. and Figure 10). This morph was almost similar to morph A(580 bp, 490 bp, 430 bp, 290 bp, 270 bp, 240 bp, 180 bp) (Apostolidis et.al.,1996). The morph obtained in this study was labelled with 'D'.

Abant populations showed the same results with those of Sümer population's. Again, the morphs exhibited by the individuals within Alakır stream were identical to each other. Genetic variation was observed among populations while no variation was observed within populations as a result of RFLP analysis of ND-5/6 region.

3.1.2.1. Mediterranean and Atlantic DNA standards

Standard DNA samples provided by Rene Guyomard which were known to belong Mediterranean and Atlantic stocks were amplified with ND-5/6 primers and then restricted with 8 restriction endonucleases. Morphs obtained were compared with the those obtained in Sümer, Abant and Alakır populations.

It was found that some morphs obtained in Sümer and Abant populations were the same with the morphs obtained in Atlantic stock. But some morphs obtained in Sümer and Abant populations did not correspond to any of the morphs obtained by the digestion of DNAs of Mediterranean and Atlantic stock. These morphs were the ones obtained as a result of *HpaII, AvaII, AluI* and *TaqI* digestion

All of the morphs obtained in Alakır populations were identical to the morphs obtained in Mediterranean stock.

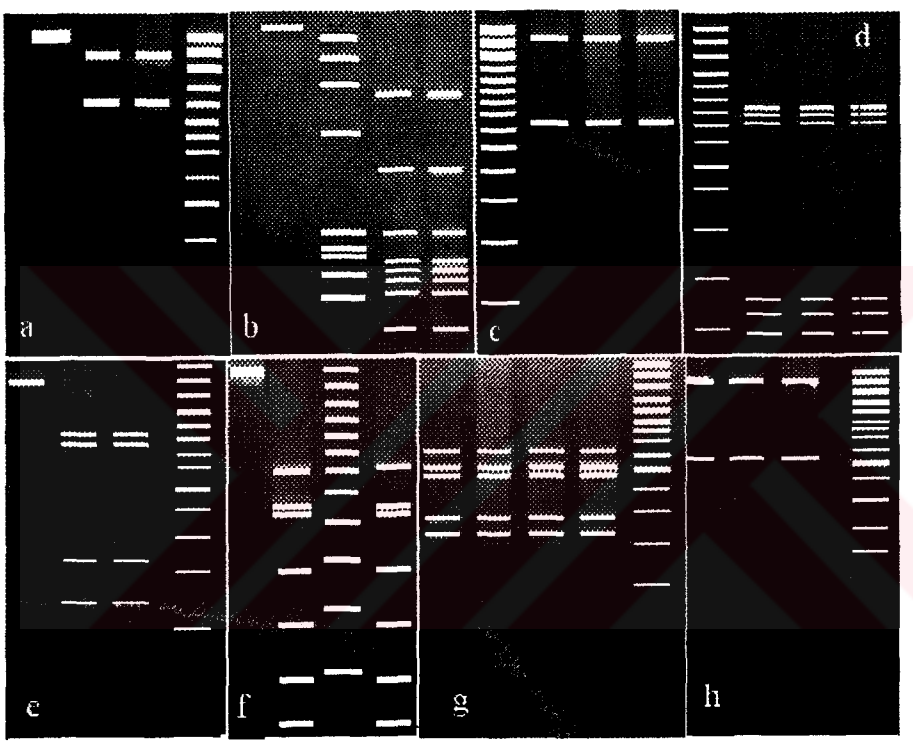

Figure 9. Restriction bands of ND-5/6 region for Sümer and Abant population after cutting with a) *AvaII*, b) *AluI*, c) *EcoRI*, d) *HaeIII*, e) *HpaII*, f) *HinfI*, g) *TaqI* and h) *XbaI*

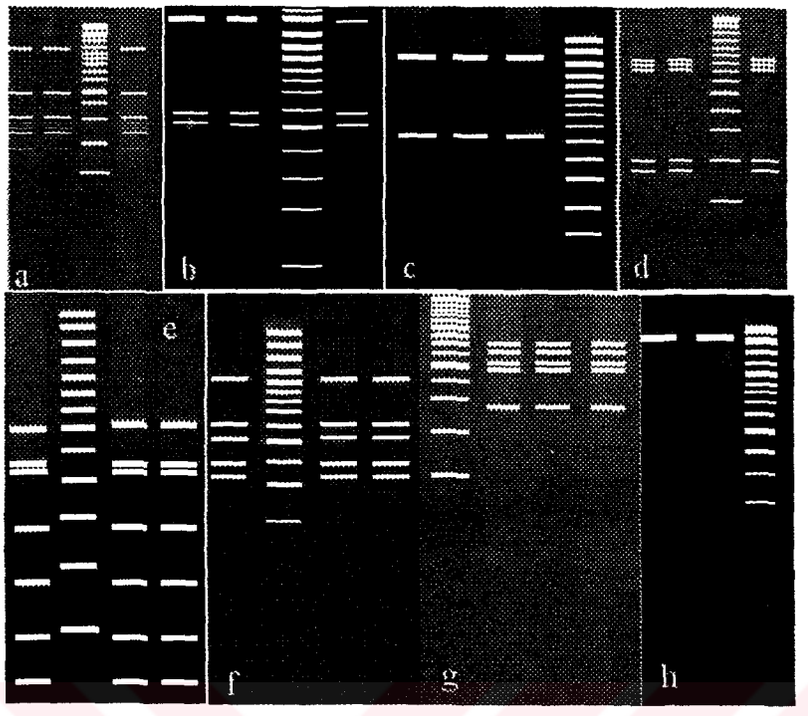

Figure 10. Restriction bands of ND-5/6 region for Alakır populations.

a) *AluI* b) *AvaII*, c) *EcoRI*, d) *HaeIII*, e) *HinfI*, f) *HpaII*, g) *TaqI* and h) *XbaI*

When the all morphs obtained were combined to form haplotypes (composite genotypes), two haplotypes were formed as a result of ND-1 and ND-5/6 region RFLP analysis of Sümer, Abant and, Alakır populations. Frequency of Haplotype 1 for Sümer and Abant populations

was 1.00 while it was 0.00 for Alakır and Gödene populations. Frequency of Haplotype 2 for Sümer and Abant populations was 0.00 while it was 1.00 for Alakır and Gödene populations (Table 7.). The combined data of ND-1 and ND-5/6 genes were analysed by the package program called DISPAN and G_{ST} (gene differentiation among subpopulations), H_T (gene diversity in total population)and H_s (average gene/haplotype diversity within subpopulations) values were found to be 1.00, 0.50 and, 0.00, respectively.

Table 7. Composite genotypes (haplotypes) and their frequencies for each locality

Haplotype	Composite Genotype*	Sample Locality		
		Alakır	Abant	Sümer
Type 1	DCADCCAGDBDDECD	0.000	1.000	1.000
Type 2	DCADCCAHEBEDFDE	1.000	0.000	0.000

* Composite genotypes are denoted by capital letters in the following order. ND-1: *AluI, AvaII, EcoRI, HaeIII,HinfI, HpaII, XbaI* . ND-5/6: *AluI, AvaII, EcoRI, HaeIII, HinfI, HpaII, XbaI and TaqI.*

Proportion of nucleotide difference between two haplotypes found to be 0.173 with 0.0001566 variance and 0.040 standard error.

Haplotype diversity between populations found to be 0.5.

MtDNA data of Turkish populations were used to construct UPGMA dendogram. It was observed that southern population Alakır (Alakır1, Alakır2, Alakır3) was differentiated from northern populations Abant (Abant1, Abant2) and Sümer (Figure 11).

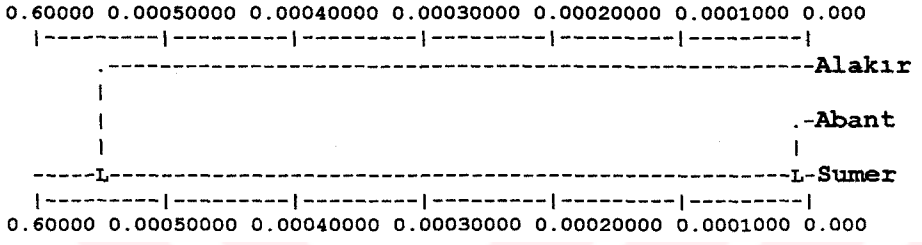

Figure 11. UPGMA dendogram for Turkish brown trout populations

3.2. Protein markers

In study done by Plan, (1999)(unpublished data), Alakır, Abant and Sümer populations were screened with respect to LDH-1, LDH2, LDH-4, LDH-5, AAT-4 and MEP-3 enzymes. Genetic distances that were calculated based on allele frequencies between these populations were summarized in Table 8. Using these genetic distances UPGMA dendogram was constructed (Figure 12).

Table 8. Genetic distances between the populations studied

	1	2	3
Alakır	0.00000		
Abant	0.19009	0.00000	
Sümer	0.11894	0.02125	0.00000

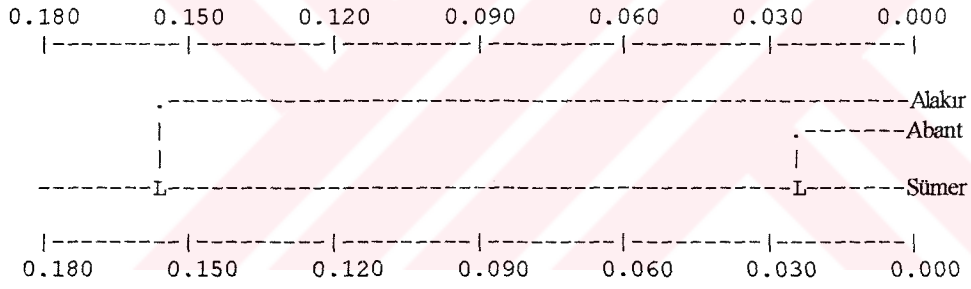

Figure 12. UPGMA dendogram constructed based on protein data

3.3. Morphological markers

In a previous study done by Yalın (1996), fish samples from Abant, Alakır and Fırtına regions were studied in terms of morphometric and meristic characters. The data of the previous study was revaluated in order to see whether the results of morphometric and meristic data were in agreement with the results obtained from mtDNA studies. The discriminant function analyses were employed for morphometric, meristic and both morphometric and meristic data sets. The results obtained were as follows:

In the case of meristic characters, the characters of main importance were the number of anal rays and the number of gill rakers. The number of dorsal rays followed these two characters

In the case of morphometric characters, the characters of main importance were minimum body length and the pectoral fin length.

In the case of both meristic and morphometric characters, the characters of main importance were the number of gill rakers, number of dorsal rays, maximum body depth and, the minimum body depth.

Mahalanobis' distance (D^2) between the populations was calculated based on each data set. It was seen that all three variable groups (morphometric, meristic and both morphometric, meristic) separated

Fırtına population from Abant and Alakır populations. UPGMA dendogram constructed based on Mahalanobis' Distances was shown in Figure 13.

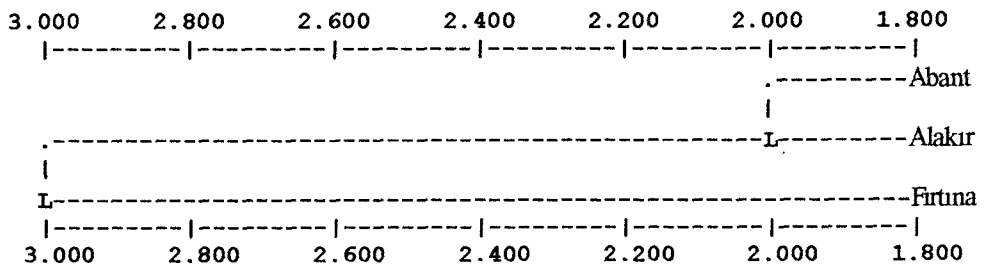

Figure 14. UPGMA dendogram constructed based on Mahalanobis distances

CHAPTER 4

DISCUSSION

In this chapter, firstly, the results of the present study obtained from mtDNA examination in different Turkish brown trout populations will be evaluated together with those obtained by other researchers in different populations from other countries. Secondly, the same results will be studied comparatively by the results based on protein polymorphism and morphological analysis of brown trout populations from Turkey.

4.1. Mitochondrial DNA

ND-1 morphs obtained in the present study were compared with that of obtained in Greek and Danish populations. The similarities were summarized in (Table9).

Table 9. Observed ND-1 morphs and their labels in terms of letters

Populations / Enzymes	Turkish populations (Present study)		Danish populations (Hansen and Loeschcke, 1996)	
AluI	620-410-410-270-200-280-140-120	(D)	660-390-290-230-200-200	(A)
AvaII	730-410-290-240-190-190-160-120-100	(C)	750-410-290-210-170-150	(B)
EcoRI	2450	(A)	Not studied	
HaeIII	620-480-290-250-200-180	(D)	660-500-280-210-200-110	(C)
HinfI	1050-790-640	(C)	1000-540-500	(A)
HpaII	790-615-615-190-150	(C)	720-520-460-180-130	(B)
XbaI	2200-250	(A)	Not studied	

All Turkish brown trout populations studied using ND-1 gene were found to be monomorphic. Only one haplotype was obtained as a result of RFLP analysis of ND-1 region. All eight populations were fixed for this haplotype. For the ND-1 region neither within group nor among group variation was detected. This result was unexpected because ND-1 region was referred as one of the variable regions on mitochondrial DNA. The restriction enzymes used in this study revealed polymorphism in some of the previous studies. In 12 populations from Danish River systems 7 ND-1 haplotypes were detected (Hansen and Loeschcke, 1996). In another study done by Hansen and Mensberg (1998), in the individuals of 24 river system eight ND-1 haplotypes were detected. In another study conducted by Hall and Nawrocki (1995) in 292 samples from Welsh, Swedish and Italian brown trout populations 7 different haplotypes were observed. But in other studies monomorphic populations in terms of one or more markers were detected. For example; in one of the studies involving sequencing of control region, it was found that all Atlantic drainage populations were monomorphic (Bernatchez et.al.,1992). In another study, two adjacent populations from north-west Scotland, which had previously found to be monomorphic for 46 protein coding loci, were studied by higher resolution techniques (Mitochondrial DNA RFLP analysis, multilocus and single locus minisatellite analysis), but no genetic variation was revealed (Prodöhl et.al., 1997). In the study conducted by Hynes et.al. (1996), all Icelandic

trout samples from north, south, east, and west coast drainages were analysed in terms of mtDNA RFLP variation and they showed only a single common haplotype.

Mitochondrial DNA, ND-1 gene was mostly studied for the populations from the north European countries. For example the samples from Denmark (Hansen et.al., 1996, 1997, 1998), from Wales and Sweeden (Hall and Nawrocki, 1995) were studied in terms of ND-1 gene and genetic variaton was observed. But there is no data available about ND-1 gene variation from the southern European countries such as France, Spain, Greece. One of the reasons may be authors from these countries might conducted preliminary studies on ND-1 gene and they may not have found any variation. Therefore, they did not continue any further.

As a result of ND-5/6 gene RFLP analysis, genetic variation was found between populations. Although the results concerning ND-5/6 gene were based on few individuals of the populations, it seems that individuals from the same water system (Alakır River, Lake Abant and Sümer River) did not show genetic differentiation. Two different haplotypes were found as a result of RFLP analysis of ND-5/6 gene. Population from the south-west Anatolia was fixed for the haplotype 1 and populations from the north-central Anatolia and the East Black Sea were fixed for the haplotype 2.

ND-5/6 morphs seen in Turkish populations were similar to morphs seen by Apostolidis et.al., 1996b and Hansen and Loeschcke (1996). But they were not exactly the same (Table 10.). Due to insertions and deletions that took place in these segments fragment sizes might be changed without causing any change in restriction sites.

The Mediterranean and Atlantic stock DNAs were amplified with ND-5/6 primers and digested with the same restriction enzymes used for screening of Turkish brown trout populations. It was seen that the morphs obtained in Mediterranean stock were the same with the morphs obtained in the populations from south-west Anatolia. Besides, some of the morphs seen in the Atlantic stock were the same with the morphs seen in population from north-central and east Black Sea of Turkey with the exception that morphs obtained as a result of *HpaII, AvaII, AluI, TaqI* digestion. The morphs corresponding to these restriction enzymes were neither present for the Atlantic nor for the Mediterranean standards. But it should be mentioned that we had just one individual from Atlantic DNA standard. If we had more than one Atlantic DNA standard that possess different morphs it might have been possible to see morphs corresponding to our *HpaII, AvaII, AluI, TaqI* morphs.

Table 10. Observed ND-5/6 morphs and their labels in terms of letters

Populations Enzymes	Turkish populations (Present study)	Greek populations (Apostolidis et.al., 1996b)	Danish populations (Hansen and Loeschcke 1996)
AluI	760-440-280-250-220-190-140-100 G) 1070-470-330-290-270-250-180 (H)	940-490-280-250-230-150-100 (A) 1090-490-280-250-230-100-20 (B)	Not studied
AvaII	1640-1130 (D) 1640-640-530 (E)	1700-810 (C) 1700-570-240 (B)	1740-840 (A) 1740-560-240 (B)
EcoRI	1870-580 (B)	2250-250 (A)	Not studied
HaeIII	930-880-760-125-90-70 (D) 930-885-760-170-130 (E)	870-710-570-170-130 (A) 870-710-570- 190-170 (C)	900-730-600-170-130 (A) 900-730-600-210-170 (B)
HinfI	650-550-420-300-270-230-190 (D)	580-490-430-290-270-240-180 (A)	960-460-310-270-240-200-150 (A)
HpaII	1200-1100-340-215 (E) 1100-620-510-340-250 (F)	1090-760-320-290 (B) 760-570-520-520-320-290 (A)	Not studied
XbaI	1720 –550 (C) 2500 (D)	Not studied	2320-250 (B) 2570 (A)
TaqI	710-560-480-250-190 (D) 800-690-550-470-260 (E)	780-580-500-310-290-230 (A)	690-600-350-310-290-180-150 (A) 840-600-350-310-290-180 (B)

G_{ST}, H_T, H_S values were calculated using package program DISPAN (Ota, 1993). G_{ST} (gene differentiation among subpopulations) was 1.00 while H_T (gene diversity in total population) and H_S (average gene diversity within subpopulations) were found as 0.50 and 0.00, respectively. In the study done by Apostolidis et.al., (1996) the gene diversity between Greek populations was found to be 1.65 in terms of combined data of PCR-RFLP analysis of D-loop, cytochrome *b* and ND-5/6 genes. In another study done by the same authors the gene diversity between populations was found 1.44 in terms of combined data of D-loop sequencing and RFLP analysis.

In the present study, number of individuals studied in terms of ND-5/6 from Alakır1, Alakır2, Alakır3 and Sümer populations were small. If they would be increased it might have been possible to see additional haplotypes. If many more populations were studied the possibility of seeing additional haplotypes would be increased. In studies done by other authors many ND-1 and ND-5/6 haplotypes were obtained. But they studied higher number of populations. For example; in Greece, Apostolidis, et.al., (1996) analysed 264 individuals from 13 different populations 11 of which were Greek populations, with respect to ND-5/6 variation. The remaining two were from France and Yugoslavia. They found nine ND-5/6 haplotypes.

Eigth of these haplotypes were fixed in 12 populations. Only in one population 3 different haplotypes were observed.

Low level of genetic variation may be due to possible bottleneck events that Turkish populations experienced. Due to its maternal inheritance and haploid nature mtDNA has ¼ the effective population size relative to nuclear genes. Hence mtDNA variation is more easily lost during population bottlenecks even mutation rate of mtDNA is higher than that of nuclear genes.

At least one population from the south-west Anatolia was found to belong to the Mediterranean phylogenetic group since it showed complete identity with Mediterranean standard DNA RFLP results. But there are three lineages in Mediterranean stock; Mediterranean (ancestral), Adriatic and Salmo trutta marmaratus (Po). It is not clear which lineage do south-west Anatolian populations belong to since we do not have DNA standards belonging to each group.

It is not clear the situation of the north-central Anatolian and east Black Sea populations. Results showed that they were similar to Atlantic phylogenetic group with respect to some RFLP results. Since they do not show complete congruence they can not be assumed as belonging to the Atlantic group. But it is known from literature that the Danube group contains Atlantic alleles as well as private alleles just belong to the Danube

group (Osinov and Bernatchez, 1996). Moreover, Black Sea basin, Aral Sea basin and Caspian Sea basins shows the characteristics of the Danube group according to mtDNA sequencing studies (Bernatchez and Osinov, 1995; Osinov and Bernatchez, 1996). Briefly, due to their geographical locations and the presence of Atlantic morphs we can conclude that the north-central Anatolian and the east Black Sea populations most probably belong to Danube phylogenetic group. To be able to decide about these populations' phylogenetic group we should also have DNA standards for the Danube, Adriatic and Marmaratus groups.

One important implication of the present study is about the status of the Abant population. The Abant brown trout population was previously defined as an endemic population by Tortonese (1954). Mitochondrial DNA results obtained in the present study did not support the endemism of the Abant population since it did not show any unique morphs different from that of observed in the other populations. This does not mean that the Abant brown trout population is not endemic. Further studies including mtDNA and nuclear genes, must be carried out.

Although the Abant population did not have any unique morph, it represents a unique appearance which makes it distinct from the others. So whether it is endemic or not it should have priority in conservation.

4.2. Protein

The results of the protein study carried out by Plan, E. (unpublished data) confirmed the results of mitochondrial DNA. Sümer and Abant populations were found closer while Alakır population was found different with respect to protein markers.

4.3. Morphology

The results were parallel when i) only meristic, ii) only morphometric and iii) both meristic and morphometric characters were used. They all showed that Abant population was closer to Alakır population with respect to characters used in this study.

Both the environmental and genetic factors effect the morphometric characteristics. Perhaps one can conclude that environmental difference between Abant and Alakır was less than the difference between Abant-Fırtına and Alakır-Fırtına.. Indeed, the water regime in Fırtına was quite different than those of the others. The water flow rate of Fırtına River was much faster than that of Alakır. This environmental characteristics might favored a shape that exhibited by Fırtına individuals. Genetic differences between these populations may be another explanation.

In many cases because of the distinct phenotypic features, some populations were excepted as subspecies of Salmo trutta. The ones found in Lake Ohrid, Lake Sevan, Lake Garda and Lake Abant were called *Salmo trutta letnica, Salmo trutta ischchan, Salmo trutta carpio* and *Salmo trutta* abanticus, respectively because of their unique appearances (Giuffra et.al., 1994; Bernatchez and Osinov, 1995; Apostolidis et.al., 1996a; Tortonese, 1954).

Yet current analyses based on genetic markers revealed that they are the populations of the same species *Salmo trutta* without exhibiting any unique alleles or haplotypes. Therefore morphometry or phenotypic characters should not be used to estimate the genetic relatedness of the populations.

4.4. Level of congruence between mtDNA, protein and morphology

The results based on mitochondrial DNA studies indicated the presence of 2 groups for the Abant, Alakır and Sümer populations. The Abant and Sümer constituted for the first group and Alakır for the second. The very same grouping was observed with respect to the protein markers. Hence, genetic markers confirmed each other. Yet, the morphological

variables both meristic and morphometric implied that Abant population is closer to Alakır rather than to Fırtına. The possible explanation for this situation may be the effects of environment as explained in section 4.3.

4.5. Suggestions for further studies

Although, mtDNA markers believed to be useful in the studies of population genetics we could not observe much variation in mtDNA of brown trout populations in Turkey. This may be due to mtDNA regions selected and the restriction enzymes used. If many regions of mtDNA had been studied, and many more restriction enzymes had been used it might have been possible to see more variation.

This was a preliminary study on mtDNA, PCR-RFLP variation of Turkish brown trout populations. In further studies, populations from East Anatolia, Agea, Thrace should be studied in terms of ND-5/6 gene. Eşen and Fırtına populations which were analysed with respect to ND-1 gene should be studied in terms of ND-5/6 gene too. Samples from Seyhan river should also be included. Since previously three specimens from these river were found to belong to a new subspecies, *platysalmo*. Another suggestion may be use of microsatellites in order to characterise the Turkish brown trout populations. Some microsatellites which can be offered may be BFRO 002 (Susnik et.al., 1997) which is considered as a characteristic for different geographically remote brown trout populations. Other

microsatellite markers should be Str 15, Str 60, Str 73, Ssosl 85, Ssosl 417, Ssosl 438 and, Ss*HaeIII* 14.20 (Hansen et.al. 1997). The latter markers were used in the studies of population genetics. Since nothing was said about their discriminatory power they should be first used in few individuals from different populations belonging to different geographical regions. Then the discriminating ones should be used.

CHAPTER 5

CONCLUSION

1- Among the two genes of mitochondrial DNA, ND-1 gene seemed to be uninformative in differentiating the brown trout populations of Turkey. Therefore in further studies on Turkish brown trout populations ND-1 gene need not be used.

2- ND-5/6 gene can be used to study the genetic relatedness of the brown trout populations of Turkey.

3- Turkish populations seemed to be exhibiting one of the least genetic variability among the studied ones.

4- Alakır population seemed to belong Mediterranean phylogenetic group.

5- Abant and Sümer seemed to belong to Danubian phylogenetic group.

6- Abant population did not exhibit any special haplotype which would be expected from an endemic population.

7- Mitochondrial DNA markers and protein markers revealed the same groups for the populations under consideration.

8- Morphometric analyses showed different resemblances between the groups than those obtained by genetic markers.

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