DETECTION OF SPECIES BOUNDARIES IN THE RANA RIDIBUNDA COMPLEX OF SOUTHWESTERN TURKEY USING MITOCHONDRIAL ND3 MARKER

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ÇİĞDEM AKIN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

JANUARY 2007

Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

DETECTION OF SPECIES BOUNDARIES IN THE RANA RIDIBUNDA COMPLEX OF SOUTHWESTERN TURKEY USING MITOCHONDRIAL ND3 MARKER

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January 2007, 135 pages

Water frogs are one of the most interesting vertebrate groups, showing great diversity and complexity in their reproductive modes, ecology and evolutionary relationships, and with many cryptic species due to high morphological similarity. For many decades, a single species, *Rana ridibunda,* has been suggested to exist in Turkey. However, the application of new morphometric, molecular and bioacoustic techniques has recently revealed the occurrence of several distinct water frog taxa in Turkey.

In this study, 340 bp long mtDNA ND3 region in 195 specimens was sequenced and subjected to phylogenetic analyses to detect geographical structure and species boundaries. Neighbor joining tree, minimum spanning network, SAMOVA and AMOVA were used to understand relationship within and among

clades. Population demography was studied through mismatch distribution and neutrality tests.

Results indicated that populations in southwestern Turkey show high diversity and strong geographic structuring. In Turkey there are four major maternal lineages, each probably representing a species: Thrace lineage represents *Rana ridibunda* Pallas 1771 in European Turkey; Ceyhan lineage indicates an unnamed taxon in Cilicia plain; South-central lineage occurs at the Lake District, Antalya, Konya and Karaman provinces and represents *Rana caralitana* Arıkan, 1988; Anatoliaca lineage (occuring in Asiatic Turkey except for central southern Turkey, Rhodes & Karpathos, northeastern Syria, and probably also Iraq and Transcaucasia) is designated either as *Rana cerigensis* Beerli, Hotz, Tunner, Heppich, and Uzzell 1994 or as a new subspecies of *R. caralitana*, based on the degree of reproductive isolation present between the last two lineages.

Keywords: water frog, mtDNA, ND3 gene, Turkey, cryptic species

ÖZ

MİTOKONDRİYAL ND3 BELİRTECİ KULLANARAK TÜRKİYE'NİN GÜNEYBATISINDA BULUNAN RANA RİDİBUNDA SU KURBAĞLARI KOMLEKSİNDEKİ TÜR SINILARININ BELİRLENMESİ

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Ocak 2007, 135 sayfa

Su kurbağları üreme tipleri, ekoloji, evrimsel ilişkilerindeki yüksek çeşitlilik ve karışıklık ve aralarındaki büyük morfolojik benzerlikten dolayı çok sayıda gizli türün bulunması sebebiyle en ilginç omurgalı gruplarından birisidir. Türkiye'de uzun yıllar tek bir türün (*Rana ridibunda*) var olduğu öne sürülmüştür. Ancak, yeni morfometrik, biyoakustik ve moleküler tekniklerin kullanılması ile birlikte farklı türlerin bulunduğu ortaya çıkarılmıştır.

Bu çalışmada, 195 bireyin 340 baz çifti uzunluğunda mitokondriyal DNA ND3 gen bölgesinin nükleotid dizi analizi çıkarılmış ve populasyonların coğrafik yapısını ve tür sınırlarını tespit amacıyla filogenetik analizlere tabii tutulmuştur. Filogenetik grupların içindeki ve arasındaki genetik ilişkileri

anlamak amacıyla NJ, MSN, SAMOVA ve AMOVA analizleri gerçekleştirilmiştir. Populasyonların geçmişteki dağılımlarını ortaya çıkarmak için mismatch dağılımı ve neutral testler uygulanmıştır.

Elde edilen sonuçlar Türkiye'nin güneybatısında yer alan su kurbağsı populayonlarının büyük bir genetik çeşitliliğe sahip olduğunu ve çok güçlü bir coğrafik yapı gösterdiğini ortaya koymuştur. Bunun dışında Türkiye'de başlıca 4 anasal soyun bulunduğu ve büyük ihtimalle her birinin farklı bir türü temsil ettiği gösterilmiştir. Trakya anasal soyu Türkiye'nin Avrupa kıtasında dağılış gösteren *Rana ridibunda* Pallas 1771 türünü; Ceyhan soyu Cilician bölgesinde yer alan henüz isimlendirilmemiş bir taxonu; Göller Bölgesi, Antalya, Karaman ve Konya illerinde bulunan güney-orta anasal soyu *Rana caralitana* Arıkan 1988 türünü temsil etmektedir. Orta-güney Türkiye, Rodos, Karpatos ve Cilician bölgeleri hariç, Türkiye'nin Asya kıtasında, kuzeydoğu Suriye ve yüksek ihtimalle Irak ve Transkafkasya'da bulunan Anatoliaca anasal soyu ya *Rana cerigensis* Beerli, Hotz, Tunner, Heppich, ve Uzzell 1994 türüne yada *Rana caralitana* Arıkan 1988 türüne ait yeni bir alttürü temsil etmektedir. Bu ayrım son iki anasal soy arasındaki üreme ayrımının derecesine bağlıdır.

Anahtar Kelimeler: su kurbağsı, mtDNA, ND3 geni, Türkiye, gizli türler

To My Family..

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and sincerest appreciation my supervisor Assoc. Prof. Dr. C. Can Bilgin for his guidance, advise, help and confidence to me throughout the this study. He had provided valuable support and opportunities for my progress in scientific area. I am grateful my co-advisor, Dr. Metin Bilgin for his invaluable help, continous encouragement for molecular part of thesis that I have learned all technical molecular details, and how can be systematically and carefully studied on the laboratory from him. Also I would like to thank Dr. Jörg Plötner for giving opportunity to study in the Alexandor von Humboldt University Sytematic Zoology Institute for nuclear marker and his deep welcoming and friendship.

I want to thank to my labmate, Banu Kaya for her inner and financal support to set up the laboratory, helps during field trips, and sincerely friendship during my troubled times and writing the thesis. Further, I am grateful, her patience and support for GIS-based softwares to construct maps so that we able to combine geographic information systems and molecular works for an interdisciplinary approach.

I would like to thank to other labmates, Didem Çakaroğulları and Senem Tuğ for the colloboration my field studies to collect samples, the arrangement of field trip, technical support and their friendships during experiment and writing thesis.

I want to thank to my rest of labmates, Hüseyin Ambarlı, Gülden Atkın, Damla Beton, İlker Özbahar, Özlem Çiğirli and Çağrı Göcek for their helps to field studies, support and friendship.

I am deeply grateful to Sezer Okay for his helps and valuable ideas during experiments and sincerely support and friendship throughout this study. And I want to thank to my other friend Aslıhan Kurt for her great support and helps during my

hard times. Also I would like to thank to the rest of friends in Prof. Dr. Gülay Özcengiz's laboratory for technical facilities and helps.

I would like to thank to Management of Agricultural and Rural Affairs in Konya and Burdur: Mehmet Türe, Osman Ali Acar, Osman Bütün, Şahin Gök, Osman Küçükavşar, Şeref Ak, Mehmet Hazır, Yusuf Ermergen, Hüdayi Alaşık, Mustafa Aydos, Management of Environment and Forest in Muğla and Antalya: Atilla Küçükala, Ramazan Kaynak, Ramazan Ongün, Dicle Tuzcu, Neylan İkizoğlu, Halil Sarıbaşak, Veyis Dorum and Turkish Bird Researh Society: Osman Erdem, Tümay Becerancı.

Also I am deeply grateful to Lebibe Pembeci, Sevilay Taşdemir, Emine Öğdem, Ayşegül Eryılmaz, Muhammet and Hilal Toker, Fatma and Hasan Çetin, Fazilet and Mehmet Çetiner, İbrahim Sözer, Yasemin Deveci, Ahmet and Bülent Akman, Yakup Çakaroğulları, Ferhat Matur, Onur Baloğlu, Hatice and Mehmet Okay for their hospitality and helps during my field trips that I had a family in each province.

I want to thank to my other labmates in the Systematic Zoology Institute: Torsten, Kai, Robert, Frank for their helps during experiments.

I thank to METU-BAP (METU-Research Fund) BAP-08-11-DPT-2002K120510 for the financial support.

Lastly, I would like to express sincerely gratitude to my father Ahmet, my mother Radife and my brother Mehmet for their endless love and support.

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Deoxyribonucleic acid: DNA

Distilled Water: dH_2O

DNA Sequence Polymorphism: DnaSP

Ethylene diamine tetra acetic acid: EDTA

Evolutionary Species Concept: ESC

Haplotype Diversity: H

Immunological Distance: ID

Jukes and Cantor Distance: d_{JC}

Klepton: kl

Magnesium Chloride: MgCl2

Mainhaplogroup: MHG

Microcomplement Fixatition: MCF

Microliter: µL

Microgram: µg

Miligram: mg

Mililiter: mL

Milimolar: mM

Minimum Spanning Network: MSN

Mitochondrial DNA: mtDNA

Molar: M

Molecular Evolutionary Genetics Analysis: MEGA

National Center for Biotechnology Information: NCBI

Neighbour Joining: NJ

Nucleus-like bodies: NLBs

Nucleotide Diversity, Pi: π

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Number of singletons: η_S

Optical Density: OD

Operational Taxonomic Unit: OTU

Over night: o/n

PCR: Polymerase Chain Reaction

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Phi: Φ

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Tris EDTA: TE

Theta: θ

Ultra Violet: UV

CHAPTER 1

INTRODUCTION

1.1. Molecular Systematics

1.1.1. Historical Development of Molecular Systematics

Systematics is the science of the diversity of organisms (Mayr and Ashlock, 1991). Linnaeus established a framework for a hierarchical system of nomenclature (binomial system) to describe and categorize biological diversity without independent of evolutionary theory (Hillis and Moritz, 1990). However, Haeckel in 1866 suggested primarily a classification based on phylogenetic relationships, and was the first to outline a phylogenetic tree covering the whole animal kingdom (Richardson and Jeffery, 2002). In the 1960s, systematics was divided into two new separate areas. One of these was numerical taxonomy or phenetics largely developed by Sneath and Sokal, (1973), which consists of application of various mathematical procedures to numerically encoded character state data for organisms under study. Phenetics mainly focus on similarity and dissimilarity among taxa and arrange them in a hierarchy.

The second area was cladistics or evolutionary systematics, of which the German systematist Hennig in 1966 was one of the pioneers. He introduced phylogenetic systematics theory to improve information content of classifications, making them the general reference system for biology. Remarkably, he also emphasized that phylogenetic reconstruction can be only based on shared derived characters (Wheeler, 2004). In 1988, Felsenstein provided a new aspect to cladistics by analyzing methods for inferring phylogenies and assessing their reliabilities.

1.1.2. Widespread Molecular Markers in Water Frogs

Phylogenetic studies require sources of inheritable variation. Nucleic acids (DNA or RNA), protein, chromosomes are important heritable markers to study genetic structure of populations or to estimate the relationship among taxa (Hillis, 1987; Hillis and Moritz, 1990). In water frogs and many other taxa, different kinds of molecular techniques were widely used to understand population structure, evolutionary processes or reveal cryptic species in previous research.

The first technique is enzyme electrophoresis, which is based on the separation of a mixture of electrically charged molecules in an electric field. The rate of movement depends on their net electrical charge, molecular size and shape. Differences among enzymes arise from changes in DNA sequences, resulting changes in the amino acid composition of the produced polypeptide. Enzyme electrophoresis reveals two distinct types of variation in enzyme polymorphism. The first type is allozyme, different allelic forms of an enzyme encoded by the same locus. The second type is isozyme, a member of enzymes that catalyze the same chemical reaction, but are encoded by different loci in the genome (Li and Graur, 1991). By means of protein electrophoresis and enzymological studies, Beerli (1994) suggested that water frogs populations in the Aegean Islands, Crete, Karpathos and Rhodes are well differentiated from the other western Palearctic water frogs at the species level. Thus, the frogs in Crete Island were described as *Rana cretensis*, and those in Karpathos and Rhodes as *R. cerigensis* (Beerli et al., 1994). Günther and Plötner (1994) found that the Italian Peninsula, Corsica and Sicily are inhabited by 3 different forms of water frogs: *Rana lessonae* which lives only in the northern part of Italy, an Italian species (*R. bergeri*), and their hybridogenetic hybrids.

Second technique is microcomplement fixation (immunological technique) which measures reactions between a soluble antigen and antibodies in dilute solution in which only high-affinity antibodies react. Complement is a sensitive indicator of the amount of antibody bounding to antigen. Therefore, altered antigenic sites

do not bind to antibody and are removed from the antigen-antibody reaction. Homologous reactions are standardized and heterologous antigens are measured relative to the homologous reaction. In this way, antigens from all species are compared and results are converted to the same unit of immunological distance (ID) (Maxon and Maxon, 1986; Maxon and Wilson, 1975). Using MCF method, Uzzell (1982) revealed the phylogenetic relationship of five western Palearctic water frogs and their divergence times.

Third is the cytogenetic technique, including two common methods: (a) Hypotonic treatment spreads metaphase chromosomes, allowing accurate identification of chromosome numbers and morphology, and (b) chromosome banding technique allows the identification of homologs (within karyotype of the same species) and homoeologs (between karyotypes of different species). By way of electrophoretic and karyological techniques, Tunner and Heppich (1982) found the presence of a cryptic species in Greece. Based on acoustic data, Schneider (1994) described this cryptic species as a new species: *Rana epeirotica*. Alpagut and Falakali (1995, 2006) showed that Aegean and Lake District water frog populations in Turkey are distinct from each other due to differences in their chromosome morphology and the number of rod-shaped bivalents in meiotic chromosomes. Mohammed et al. (1997) found that water frog populations from Bahrain, Saudi Arabia, and Egypt do not belong to different species.

Fourth is the microsatellite marker, which has become an effective and widely applicable technique in recent years. A microsatellite is made up of tandem repeats of short sequence motifs (no more than six bases long). They are PCR-based markers and highly polymorphic (Tautz, 1989) especially, when they are long and uninterrupted. Rate of mutation of microsatellites are high. Therefore, they are useful genetic markers identity studies, population studies, linkage analysis and genome mapping (Tautz, 1989; Goldstein and Schlötterer, 1999). By means of microsatellite loci and mating system analyses, Austin et al. (2003) found the presence of female-biased dispersal in the bullfrog (*Rana catesbeiana*). In another study, Hoffman et al. showed effective population size and temporal stability of genetic structure in *R. pipiens* populations. In a following study,

using microsatellite-based method, Christiansen (2005) showed that genotype and ploidy can be accurately detected in *R. esculenta* complex and in museum specimens, embryos, and saliva samples from protected populations.

1.1.3. Advantages of DNA-based Taxonomy over Traditional Taxonomical Approaches

Taxonomy is the theory and practice of classifying organisms (Mayr and Ashlock, 1991). DNA-based taxonomy, especially DNA sequencing, have several advantages over the traditional methods to resolve problems at a taxonomical level, to understand population level processes and phylogeny, where morphological methods alone remain inconclusive. These advantages are discussed below.

1. Molecular techniques are reliable for defining species boundaries at five conditions. First, when two species are sympatric or parapatric but they are morphologically too similar to obtain separate species status (Donellan and Aplin, 1989). Second, when two allopatric populations are morphologically different, but their status as separate species is not clear. Third, when two parapatric populations are morphologically distinct, but they show clinal variation or wide range of hybridization. Fourth, when two morphologically distinct forms may show polymorphism within a single interbreeding population (Titus et al., 1989). Fifth, when morphologically similar asexual species forms arise independently from sexual species (Hillis and Moritz, 1990).

2. Development of web taxonomy makes the vast of information universally accessible and free. Such information includes a traditional description of each taxon, the location of type material, keys for formal description, photographs, other illustrations for many groups and also DNA sequences for taxa. Furthermore, the presence of a link between a species description and increasing amount of scientific literature, including early

papers on its taxonomy and biology, gives a chance to the ecologist, conservationist, amateur naturalist to describe animal and plants (Charles and Godfray, 2002).

3. DNA-based taxonomy is a new scaffold for accumulation of taxonomic knowledge and a reliable tool for species identification and description (Tautz et al., 2002). Unique reproducibility of DNA sequences prevents duplicate descriptions (Lee, 2002). Besides, collection and storage of extracted DNA samples are technically easy. DNA is very stable and any sample can be separated into multiple subsamples, which can be sent to other museums as backups, which requires for many projects in different laboratories studying the phylogeny or phylogeography of species (Tautz et al., 2002; 2003). At the pylogenetic spectrum, many gene sequences evolve at a different rate, providing a record of a evolutionary history from very recent to ancient times (Hillis, 1987).

4. The comparative data obtained from molecular characters is useful for phylogenetic construction when the characters must show heritable variation. Thus, in terms of genetic variation, molecular characters are less affected by environmental influences than morphological characters (Hillis, 1987).

5. DNA barcode is a short sequence of standardized gene regions, providing rapid, accurate and automatable species identification (Hebert et al., 2003; 2004a; 2004b; Hebert and Gregory, 2005). DNA barcoding can be used substantially to identify cryptic species, which are morphologically inseparable including many unknown taxa (Hebert, 2004b, Proudlove and Wood, 2003).

6. The barcoding is also an effective technique to identify micro and meiobiota which are abundant, productive and saprophytic, in other words, having a key function in the ecosystem. However, when their microscopic size is taken into account, it is really difficult to distinguish

their morphology without DNA barcoding technology (Blaxter and Floyd, 2003; Blaxter et al., 2005).

7. DNA taxonomy is considerably used to study hybridization, which is a problem when inferring phylogenetic relationship, especially when the hybridizing species occur sympatrically over large areas. Hence, it provides more characters than morphology to differentiate hybrid individuals. In addition, it helps to measure the extent of hybrid zones and introgression of genes between species (Masta et al., 2002; Johannesen et al., 2005).

8. DNA taxonomy is a key component of conservation biology, particularly for conserving taxonomically complex groups (TCGs), which have some form of uniparental reproduction (e.g. self fertilization, hybridogenesis). In TCGs, hybridization occurs to some degree among their members, which produces genetically different individuals with more than one ploidy level, hence their simple classification is not easy using traditional methods (Ennos et al., 2005). In contrast, thanks to DNA taxonomy, the structure of different population systems with distinct ploidy levels can be clarified and their conspecific individuals can be distinguished (Plötner et al., 1994).

9. The use of DNA sequences is a useful phylogenetic tool. Firstly, it provides unlimited number of characters for morphologically conservative groups. Secondly, sequence characters allow the comparison of any taxa, providing the development of higher-level phylogenies. Thirdly, a molecular phylogeny may answer better a variety of questions related to the ecology, life history, and biogeography (Dawood et al., 2002).

1.2. Speciation and Species Concepts

1.2.1. What is a species ?

For a taxonomist, defining a species is not easy, because there are many debates to explain whether species are real or a human–made definition and even species are real which concept is better to define a species objectively. However, evolution and speciation are continuous biological processes. Many studies are carried out to unravel these processes and their results based on discrete groups as a species (Helbig et al., 2002). Indeed, species is treated as real in different disciplines. For instance, naturalists label their specimens, population geneticists measure the degree of genetic variation within species, taxonomists reconstruct phylogenetic relationships among species, ecologists calculate species diversity (Coyne and Orr, 2004). Consequently, species are the fundamental unit for much of ecology, taxonomy, conservation biology, evolutionary genetics (Barton, 2001), and understanding the causes of biological diversity and preserving it require to identify species accurately and agreeably (Hey, 2001).

Over the past century, many authors have proposed their own definitions of what is a species, which led to many controversies. Fundamentally, the species concepts are divided into three categories: Biological Species Concept (BSC), Phylogenetic Species Concept (PSC), and Evolutionary Species Concept (ESC).

1. **Biological Species Concept (BSC):** In 1942, E. Mayr defined that a species is a group of interbreeding natural populations that are genetically isolated from other group by reproductive isolating mechanisms (Quicke, 1993).

2. **Phylogenetic Species Concept (PSC):** In 1983, J. Cracraft first defined PSC, that a species is the smallest group of a sexually reproducing organism that possesses at least one diagnostic character in all group members but it is absent in all close relatives of the group (Quicke, 1993).

3. **Evolutionary Species Concept (ESC):** It was put forward by G.G. Simpson as a single linegae of ancestral descendant populations which maintains its identity and has its own evolutionary tendencies and historical fate (Wiley, 1978)

In 1931, Mayr also introduced the superspecies concept; that is, a monophyletic group of allopatric or nearly allopatric taxa that are believed to have evolved to the species status. A superspecies is composed of a monophyletic group of allospecies (a geographically separated) or semispecies (connected by a hybrid zone) (Helbig et al., 2002). Semispecies is referred to those populations that have not fully speciated and gene flow is still present among semispecies, but not to a great extent as among conspecific populations. To put it in a different way, semispecies occupy an intermediate category between species and subspecies. In contrast, allospecies is designated as geographic isolates that have clearly reached the species level, but have remained a member of a superspecies (Mayr and Ashlock, 1991). Indeed, the superspecies concept is important to clarify zoogeographic studies, to understand evolution especially recently evolving taxa and widely used in systematic works, checklists to show patterns and processes in speciation that are not clear (Amadon, 1966).

Research shows that many subspecies separated by hybrid zones do in fact differ at multiple morphological, behavioural, and genetic characters (Mallet 2001). For instance, the toad *Bombina bombina* meets its relative *Bombina variegata* across a broad front in Europe. The two forms hybridize freely in the contact zone, and therefore, should really be classified as members of the same species under BSC, although the hybrids can be shown to suffer some inviability (Barton and Hewitt, 1985).

However, such well-defined forms are usually placed in separate species in spite of the fact they have not fully "speciated." The two *Bombina* taxa, for example, differ strongly in call, morphology, skin thickness, the sizes of water bodies used for breeding, and egg size, as well as mtDNA and protein sequence. The levels of differentiation suggest that those taxa have evolved separately for many millions of years. This situation of multiple character changes has now been

shown to be true across many examples of subspecies as well as species separated by hybrid zones. Gene flow can be shown to be almost completely blocked by hybrid zones such as these, even if hybridization is frequent (Barton and Hewitt, 1985; Jiggins & Mallet 2000).

1.2.2. Speciation (How does a new species form ?)

Speciation is divergent evolution resulting into two species from an initial ancestral species (Loses and Glor, 2003). During this process, many factors and mechanisms take a role with diverse level effects based upon conditions. The factors include predominantly environmental parameters such as, biotic and abiotic elements of a habitat (e.g. climate, resources, physical structure), interactions with other species (resource competition, predation, mutualism, and various forms of interspecific interference) (Schluter, 2001), whereas the mechanisms mainly consist of prezygotic and postzygotic isolation, fundamental requirements for different types of speciation processes. Mate preference, secondary sexual traits, mating signals, assortative mating (Panhuis et al., 2001), sperm and egg incompatibility (Johannesson, 2001) and habitat isolation are the most powerful parameters leading to the prezygotic isolation mechanism, which is the primary cause of reproductive isolation. On the other hand, the postzygotic isolation mechanism includes hybrid sterility, inviability, the accumulation of incompatibilities between alleles in different lineages (high frequency of developmental abnormalities) (Turelli et al., 2001), chromosomal rearrangement in populations (which reduces gene flow between species by suppressing recombination and extending the effects of linked isolation genes) (Rieseberg, 2001). In addition, ecological selection against hybrids (an intermediate phenotype of hybrid is less effective in capturing prey in the wild, and an intermediate defense mechanism makes hybrids more susceptible predation and parasitism) (Schluter, 2001), a rapid evolutionary divergence of animal genitalia (Panhuis, et al., 2001), and high rates of nonsynonymous:synonymous nucleotide substitution ratios in sex related genes (genes involving in mating behavior, fertilization, spermatogenesis or sex

determination) among closely related species are the other factors, giving rise to postzygotic isolation (Civetta and Singh, 1988). Sympatric, allopatric (vicariant and peripheral), and parapatric speciation are the primary speciation models.

Sympatric speciation: In this model, a population splits into two, but they can still coexist at the same locality. In addition to premating isolation mechanisms, distruptive (divergent) selection on morphological, physiological, behavioral, sexual traits and on habitat choice lead to reproductive isolation (Schluter, 2001). Hence, they remain distinct because of little or no gene flow and reproductive isolation (Helbig et al., 2002). Nevertheless, recombination breaks up either closely linked genes involving reproductive isolation and the evolution of assortative mating. As a consequence, reproductive isolation and the ability to coexist must evolve together to prevent the effect of recombination and competetive exclusion during sympatric speciation (Coyne and Orr, 2004).

Allopatric speciation: In this model, pre- and postzygotic isolating mechanisms are again important sources for genetic divergence and reproductive isolation like in sympatric and parapatric speciation. Allopatric divergence is directly related to adaptation to a particular habitat, a resource, a mating site or the sexual isolation (effects of ecological differences between habitats on either male traits or female preference), extrinsic isolation (hybrid viability and fertility), intrinsic isolation (incompatibility, developmental problems independent from environment), genetic drift, and mutation (Turelli et al., 2001). Allopatric speciation is split into two types: Vicariant speciation and peripatric speciation.

In vicariant speciation, the geographic range of a species divides into two or more large, isolated populations followed by reproductive isolation. A variety of geographical and climatic events such as glaciation, formation of mountains, continental drift, climate change, and extinction of an intermediate population may lead to geographical separation, preventing the gene flow among the populatios. Subsequently, different types of selection act not only on different habitat types, but also sexual traits. In addition, mutation, fixation of different genes in different populations by genetic drift (primary cause of genetic incompatibility) increase in initial genetic divergence. As a result of these

events, isolation barriers provide species to remain distinct until they encounter each other with secondary contact. When they come into a contact, they maintain their evolutionary identities, because selection prefers any trait that makes hybridization or interbreeding unlikely (Baker, 2005; Coyne and Orr, 2004).

In peripatric speciation, after a small geographic habitat with a population isolates from a mainland population, initially colonization occurs by rapid adaptation to new habitats, which is followed by rapid speciation due to strong selection. Meanwhile, individuals with a small part of ancestral genetic variation are subjected to genetic drift, which lead to the fixation of new advantageous or neutral alleles. Finally, these new alleles lead to incompatibility between the ancestor and descendant populations. Also, they cause loss of genetic variation and an increase in inbreeding in descendant populations, providing material for sexual selection (male becomes less attractive against female derived from ancestral population) (Ödeen and Florin, 2002; Coyne and Orr, 2004).

Parapatric Speciation: In this model, populations are adjacent to each other, reproductively isolated, and do not require geographic barriers. During this process, initially, each population is diverged in a new ecological niche and locally adapted to a new habitat, in which selection favors reduced migration and gene flow among populations. In the meantime, change in a geographic gradient or isolation by distance lead to accumulation of genetic differences (e.g. sudden change in allele frequency at one locus or presence of different alleles in different places [Turelli et al., 2001]), and local adaptations cause genetic divergence (Gavrilets et al., 1998); hence, they become the beginning of reproductive isolation. Afterwards, selection on sexual traits (male traits and female preferences) (Lande, 1982) and evolution of assortative mating in the locally adapted populations accelerate the reproductive isolation among adjacent populations. Besides, recombinant fit individuals resulting from parental hybridization can easily be adapted and coexist together with their parental individuals, providing some degree of reproductive isolation (Turelli et al., 2001; Coyne and Orr, 2004).

1.2.3. Introgression and Hybrid Zones

Introgression is the incorporation of allochtonous genes from one group of populations to another, resulting in groups of recombinant individuals. Detection of these hybrids are obtained through the analysis of set of characters such as morphology, chromosomes, allozymes, mtDNA, nuclear loci, etc because the proportion of characters will be influenced by selection, drift, genetic and other environmental factors. Therefore, parental contributions may not be present for all characters. The result of this process might be origination of new species via combination of parental characters. Further, extensive introgression is more common in the habitats which are considerably influenced by human disturbance, thus; it can be used to track environmental changes, causing the increase in level of variability (Dowling and Secor, 1997).

Hybrid zones are narrow regions in which genetically different populations meet, mate, and produce hybrids, containing many independent genes, characters or combinations of parental features. Modes of zones in spatial areas are divided into 2 classes: (a) Dispersal-independent zones, in which selection is stable at each locality and dispersal is little or negligible. (b) Tension zones, in which there is a dispersal/selection balance and differences in environment or selection against heterozygote or recombinant genotypes maintains a stable zone. Briefly, tension zones tend to minimize their length by response to local environmental conditions (Barton and Hewitt, 1985).

A cline is continuous geographic change in the frequency of a single gene, chromosome or character. In hybrid zone, clines at different loci and characters such as morphology, mtDNA, nuclear DNA is considerably coincident (the center of the clines in the same position) and concordant (similar width and shape) (Barton and Hewit, 1985). Nevertheless, selection pressure on several loci and characters are different, so the distinct rate of introgression through hybrid zone might result multiple clines, which do not coincide or are concordant with each other. The position and width of clines are maintained either by exogenous selection due to environment (Haldene, 1948) or by endogenous selection such as

selection against heterozygotes and frequency dependent selection against rare genotypes (Mallet and Barton, 1989; Johnson et al., 1990; Dasmahapatra et al., 2002).

1.3. Water Frogs

1.3.1. General Information on Water Frogs

Rana is a speciose and succesful genus of the amphibians. Nearly, more than 250 *Rana* species have been identified on a great part of the world, except the polar regions and oceanic islands, including North and South America, Asia, Europe, Australia, Madagascar, Africa and New Guinea. Within their range, they mainly inhabit marshes, freshwater streams, ponds and lakes in tundra, temperate coniferous, deciduous forest, grassland, desert, semitropical cloud forest and tropical rain forests (Hillis and Wilcox, 2005). Adult diet generally includes insects and their larvae, earthworms, and other invertebrates (Berger, 1988; Pough et al., 2001). Water frogs are distinct from brown frogs by means of paired external lateral vocal sacs in males, extension of webbing of feet to the tips, absence of a black face mask from the eye to the tympanum, and the presence of dark mottling on the inner thigh surfaces (Beerli et al., 1994). Furthermore, they are easily distinguished from toads by presence of two bulging eyes, strong, long and webbed limbs for jumping, smooth skin (preferably moister environments), while toads have a stubby body with short limbs for walking, warty and dry skin (drier climates), paratoid glands behind the eyes (http://allaboutfrogs.org/froglnd.shtml) (Figure 1-1).

Figure 1-1. A water frog in an irrigation canal. (Eber Lake, 18.09.2005; Çiğdem Akın).

Their skin has important role in enviromental physiology, defense (secretions and colors), mate selection and courtship behaviors (sexual displays, nuptial pads), locomotion (toe pads and webbing). It is smooth, soft, moist, and regulates oxygen intake in respiration. The skin has many granular glands, producing poisonous secretions against predators. During the mating season, male frogs develop mucus glands on their hands called as nuptial pads. They are a keratinized epithelium, which help males to embrace females during amplexus (Pough et al., 2001).

Frogs produce different types of calls: Mating calls, territorial calls, and transitional calls. During the mating season, male frogs produce a mating call to attract females, which has species-specific features. It is reliably distinct to bring the two sexes of a given species together. Thus, it is quiet useful not only for intraspecific auditory communication, but also informative to understand the kinship relations among water frogs (Schneider and Sinsch, 1992).

Their main habitats are small and shallow waters, forest ponds, peat bogs (e.g. *Rana lessonae* mainly hibernates on land because of higher resistance to low temperature. By means of small body size and short legs with large callus internus, it can easily dig in the soil.), or large and deep water: rivers, channels, lakes, old river-beds, large gravel-pits and clay-pits (e.g. *R. ridibunda* hibernates in water, being sensitive to oxygen deficiency and lacking adaptations to dig in

the soil because of a very small callus internus and long hind limbs) (Berger, 1988; Rybacki and Berger, 1994) (Figure 1-2).

Figure 1-2. A reedy stream bed, a water frogs habitat (Edirne - Yağcılı, 31.08.2005; Çiğdem Akın).

Frogs are ectothermic animals; that is, their body temperature depends on environmental conditions: climate and seasonal changes. In winter, they hibernate in the mud of the bottom of pools and streams or on the small burrow in the soil. In the spring, male frogs locate a suitable breeding site and produce the distinctive mating call to attract females. When eggs are mature, females enter into water and are clasped by males, called amplexus. Then males release the sperms over the eggs to fertilize them. The eggs are attached to vegetation in water. Following fertilization, development starts immediately. At 7 to 10 days, a tadpole hatches from the protective jelly coat, having a clear head and body with a tail. During metamorphosis, body organization is changed internally and externally (resorption of tail and burst of limbs). They become sexually mature in 2-3 years (for males) or 3-4 years (for females).

The *Rana* genus has also an economical value. It is commonly used in scientific research and educational purposes since they are easy to obtain, handle, and dissect at the laboratory. Additionally, they are raised and exported to many

countries as a food source. For instance, in each year approximately 1300 tone of frog legs are exported from Turkey to mainly France, Italy, Belgium, and Luxemburg. Also, they are consumed considerably in China, and America.

1.3.2. Western Palearctic Water Frog Complex

Water frogs are one of common amphibians in the Western Paleractic region. During recent years, they attracted the attention of many researchers because of complex evolutionary relationships, unusual features of their population genetics (Joerman et al., 1987) and presence of many cryptic species (Tunner et al, 1982; Plötner et al., 2001). Especially, the existence of different hybridogenetic systems in the specific population types with particular genotypes is a well-representative indicator of the great variability and complexity of reproductive modes in water frogs (Plötner and Schmeller, 2001). Additionally, presence of a high morphological similarity, lack of diagnostic morphological characters, presence of interspecific hybrids, the limited species-specifity of mating calls in closely related water frogs make the situation much more complicated (Plötner et al., 2001), and species in this group are highly diverse in ecology, physiology, behaviour (Hillis and Wilcox, 2005). Therefore, the water frog complex is a good model to study genetic diversity, biodiversity, biogeography, ethology, phylogenetic relationships, speciation and radiation events (Nevo et al., 1988; Beerli, 1994; Beerli et al., 1994; Plötner et al., 2001).

Hybridogenesis is the most interesting and complicated reproduction mode in water frogs. Hybridogenetic lineage is designated as klepton, which was designated as "kl" between the genus name and the species name (Dubois and Günther, 1982). At this system, the hybrid of two parental species (e.g. A and B) is able to reproduce by backcrossing to one of its parents. Before gametogenesis is completed, one chromosome set is lost during gametogenesis; hence, only one set remains (A or B). However, the hybrid has two set chromosomes (AB) in its somatic cells. Then, the hybrid with A in its gonads can backcross with B or vice versa (Graf and Mullar, 1979). Likewise, *Rana* kl. *esculenta* is a natural hybrid

of crosses between *R. ridibunda* and *R. lessonae* (Berger, 1966; 1967; 1968). It reproduces by hybridogenesis and lives with one of parental species together. During reproduction, only chromosomes of one parent either *ridibunda* or *lessonae* genome is transmitted, while the chromosomes of the other parent are eliminated before gametogenesis is completed (Tunner and Heppich, 1981; 1982). The chromosome elimination occurs by a formation of nucleus-like bodies (NLB) from the interphase nuclei (Ogielska, 1994). In this mating system, apart from diploid esculenta populations, triploid *esculenta* populations having RRL or LLR genotypes exist (Günther et al., 1979).

In the last 35 years, water frog taxonomy has undergone important changes; the status of many taxa - either species or subspecies - have been corrected, renewed or new taxa have been described. Primarily, detection of the hybrid nature of *R. esculenta* and its separation from *R. lessonae* (Berger, 1966; 1967), led to much more investigation and new findings in water frog systematics (e.g., the identification of new cryptic species: *Rana shqiperica* in Yugoslavia (Hotz et al., 1987), *R. epeirotica* in Greece (Schneider et al., 1984)). Remarkably, the development of molecular techniques have provided a powerful tool for evolutionary genetics with maximum information and resolution on whole genome (Uzzell et al., 1994) and a great value on systematic studies (Plötner et al.,2001), which led to an increase in the number of species, following their removal from the heterogenous *R. ridibunda* taxon and description as new species. Recently, water frogs have been considered to consist of many biological species or semispecies and some hybridogenetic linegaes (Günther, 1994). According to Dubois and Ohler (1994), Berger (1999) and Günther (1999), there are now 12 available taxa for frogs of the subgenus *Pelophylax* in the western Palearctic region. They are the following taxa:

1- *Rana bedriagae* Camerano, 1882 (distribution: western Syria, Jordan, Palestine and northern Egypt.).
2- *Rana bergeri* Günther, 1986 (distribution: Peninsular Italy, Corsica and Sicily).
3- *Rana cretensis* Beerli, Hotz, Tunner, Heppich, Uzzell, 1994 (distribution: Crete, Greece).

4- *Rana epeirotica* Schneider, Sofianidou, Kyriakopoulou-Sklavounou, 1984 (distribution: Northwest of Greece and the southern parts of Albania).
5- *Rana lessonae* Camerano, 1882 (distribution: From France to Volga River basin, Russia).
6- *Rana perezi* Seoane, 1885 (distribution: On the Iberian Peninsula and in southern parts of France).
7- *Rana ridibunda* Pallas, 1771 (distribution: From eastern France to western China).
8- *Rana saharica* Boulenger in Hartert, 1905 (distribution: Northern Africa).
9- *Rana shqiperica* Hotz, Uzzell, Günther, Tunner, Heppich, 1987 (distribution: Southwest of Yugoslavia and the northern parts of Albania; taxonomic position is not clear).
10- *Rana terentievi* Mezhzherin, 1992 (distribution: Tajikistan).
11- *Rana cerigensis* Beerli, Hotz, Tunner, Heppich, Uzzell, 1994 (distribution: Karpathos, Rhodes; taxonomic position is not clear).
12- *Rana kurtmuelleri* Gayda, 1940 (distribution: Southern Yugoslavia, Albania, and Greece; taxonomic position is not clear).

Furthermore, there are 3 klepton species as follow:
1- *Rana* kl. *esculenta* Linnaeus, 1758 (distribution: From France to Volga River basin, Russia).
2- *Rana* kl. *grafi* Crochet, Dubois, Ohler, Tunner, 1995 (distribution: from southern France to northeastern Spain).
3- *Rana* kl. *hispanica* Bonaparte, 1839 (distribution: Peninsular Italy, Corsica, and Sicily).

1.3.3. Taxonomical Studies in Water Frogs of Turkey: From Past to Present

Water frogs are very widespread in Turkey. However, until the early 1990s, there were few taxonomical studies concerning water frogs, which were rather general and superficial. For many years, it was thought that Turkey had a

single species of water frog, *R. ridibunda,* Pallas 1771 (Bodenheimer, 1944; Başoğlu and Özeti, 1973; Andren and Nilson, 1976). Nevertheless, the presence of large specimens with brilliantly orange colored spots on their ventral parts in Beyşehir was mentioned (Bodenheimer, 1944; Başoğlu and Özeti, 1973).

In 1992, Schneider et al. described a new species, *Rana levantina*, based on bioacoustic analysis, from southwestern part of Asia (including Turkey). However, according to the priority rule, it is now considered a junior synonym of *R.* bedriagae Camerano 1882 (Dubois & Ohler, 1994; Beerli, 1994). Moreover, Beerli (1994) and Jdeidi et al. (1999) showed that Anatolian and Balkan frogs have distinct allozyme markers. Furthermore, when comparing with *R. ridibunda* from type locality and Balkans, differences in respect of mitochondrial DNA markers (Plötner, 1998; Plötner et al., 1999) and features of mating calls (Jdeidi et al., 1999, 2000; Schneider et al., 1999) were detected. Berger's crossing experiments (unpublished) showed that F1 and B1 offspring of *R. bedriagae* x *R. ridibunda* or *R. bedriagae* x *R. kurtmuelleri* crosses were infertile or had developmental abnormalities. Therefore, water frogs from Anatolia was accepted as *R. bedriagae,* Camerano 1882 by means of morphometric and bioacoustic data (Schneider et al., 1999; Jdeidi et al., 1999).

Rana ridibunda caralitana was firstly described as a new subspecies by the presence of orange colored maculation on the dirty white ventral part of specimens (Arıkan, 1988). By means of morphometric and serological methods, the same author in 1990 suggested that populations living in Anatolian Lake District (Beyşehir, Suğla, Eğirdir, Akşehir, Eber), Marmara Region (Ulubat, Manyas, Sapanca, Abant), Ordu (Ulubey) and Malatya could be accepted as separate forms. Karyologically, Alpagut and Falakalı (1995, 2006) showed that the Lake District and Aegean populations differ in the location of centromers,the presence of secondary constriction on chromosome in mitosis, and the number of rod-shaped bivalents in meiosis. Jdeidi et al. (1999, 2000), using morphological, allozyme and bioacoustic analyses, suggested three separate groups in Turkey, where the form *caralitana* Arıkan 1988 inhabited the Lake District, *R. ridibunda* Thrace and *R. bedriagae* the rest of Anatolia. Consequently, *R.*

caralitana was suggested to be raised to species status, which was also supported by Plötner and Ohst's mitochondrial DNA studies (2001).
According to mitochondrial DNA analysis, Plötner et al. (2001) also showed the presence of three Anatolian lineages and a Cypriote lineage. The first lineage is distributed from western Turkey to the Caucasus, and also occurs in Rhodes, Karpathos, and central Syria on the River Euphrates. The second lineage includes the southwestern part of Turkey (Lake District population). The third lineage represents only Ceyhan (Adana). Furthermore, it was suggested that neither Anatolian nor Cypriote water frogs belong to *R. bedriagae* which is restricted to western Syria, Jordan, Palestine and northern Egypt.

By comparing specimens with those from the type localities of *Rana bedriagae* (Damascus, Syria) and *R. ridibunda* (Atyrau, Kazakhstan) morphometrically, Sinsch et al. (2002) suggested the presence of two morphs in Turkey: Mostly *R. bedriagae*, and a second morph only present at Ulubey near Ordu. This second morph is considered not conspecific to either *R. bedriagae* or *R. ridibunda*, but is more similar to *R. bedriagae* morphometrically. In the same study, Anatolian Lakes District population was suggested as *R. bedriagae caralitana.*

1.3.3.1. The *caralitana* form

Class: Amphibia

Order: Anura

Suborder: Neobatrachia

Superfamily: Ranoidea

Family: Ranidae

Genus: Rana

Subgenus: Pelophylax

Original Name : *Rana ridibunda caralitana* Arıkan 1988.

Type Locality : Beysehir Lake; Konya, Turkey (37 40 N, 31 30 E).

Figure 1-3/4. The view of ventral part (on the left) and dorsal part (on the right) of the *caralitana* form (Konya - Beyşehir, 22.08.2005; Çiğdem Akın).

The ground color of dorsal part is light brown, covered with dark brown irregular maculation. Ventral part is dirty white, covered with orange color maculation (including the ventral parts of their heads and extremities) (Figure 1-3/4).

The distribution of this frog is reported to extend to Ivriz - Konya in the east, Aksehir Lake and Karapinar - Konya on the north, Cardak - Denizli on the west, and Elmali (Girdev Plateo), Korkuteli - Antalya and Tınaztepe - Konya on the south (Atatür et al., 1889 -1990; Arıkan et al., 1994; Arıkan et al., 1998; Budak et al., 2000; Jdeidi et al., 2001; Kaya et al., 2002; Düşen et al., 2004; Tosunoğlu et al., 2005) (Figure 1-5).

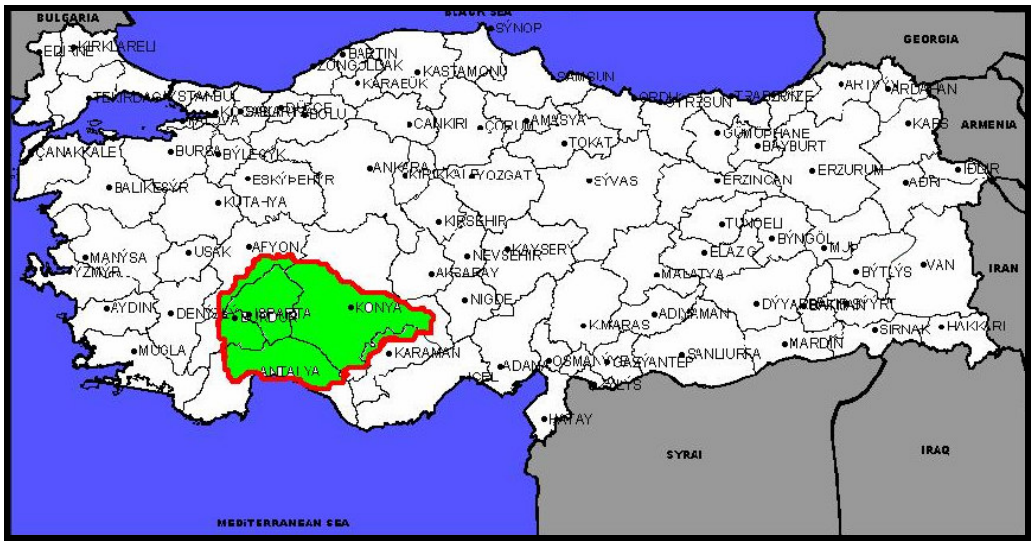


Figure 1-5. Green area enclosed with red line showing the known range of *caralitana* form according to recent studies.

1.4. Mitochondrial DNA and ND3 marker

Mitochondrial DNA of amphibians are highly conserved and quiet compact. The gene content, base composition and codon usage of mtDNA are typical to vertebrate patterns (Sano et al., 2004; 2005). The genome is 16 - 23 kbp in length, and contains genes for 22 tRNAs, 2 rRNAs (12S and 16S rRNA) and 13 proteins (ATP 6, ATP 8, CO I, CO II, CO III, Cyt b, ND1-6, which are involved in electron transport and oxidative phosphorylation on the inner membrane of mitochondria (Avise, 2000) (Figure 1-6). Besides, non-coding D-loop region with many copies of tandem repeat sequences and several short regulatory elements has signals for the replication and transcription (Sumida et al., 2001). Although gene order is highly conserved in mtDNAs, many amphibians were found with a rearranged gene order. These arrangements are mainly present in tRNA genes, D-loop region and translocation or absence of some protein coding genes. For instance, presence of four tRNA genes arrangements in *Rana nigromaculata* (Sumida et al., 2001), an accumulation of repetitive sequences in the D-loop

region, the positioning of four tRNA genes and ND5 gene between D-loop and 12S rRNA in *Buergeria buergeria* (bell-ring frog) (Sano et al., 2004), an exchange positions of two tRNA genes, lack of ATP8 and ND5 genes in *Polypedates megacephalus* (a tree frog) (Zhang et al., 2005), the inclusion of two control regions and accumulation of repetetive sequences in these regions, the presence of 4 tRNA gene arrangements in *Rhacophorus schlegelii* (Schlegel's tree frog) (Sano et al., 2005), the arrangements of some genes and gene regions, the presence of two distinct tRNAs and a pseudogene for methionine and two control regions with high sequence homology in *Mantella madagascariensis* (Malagasy poison frog) (Kurabayashi et al., 2006).

The most striking features of mtDNA include the maternal inheritance, its high copy number with cytoplasmic location in most cell types, lack of recombination (although recently, it has been suggested the evidence for recombination between heterologous mtDNA in humans (Kraytsberg et al., 2004)), higher mutation rate than nuclear genes, and rapid evolution (due to an inefficient mtDNA replication complex, the low functional constraints, mutation with a little or no effect on the fitness of the organism) (Brown et al., 1979; Saccone et al., 1999; Avise, 2000; Pakendorf and Stoneking, 2005).

Remarkably, base composition of mtDNA is substantially variable, and its each component evolves at different rate (Liu et al., 2000). For example, 12S and 16S ribosomal RNA genes evolve slowly, adequate for resolving older divergences (Mindell and Honeycutt, 1990), and have a low variation rate (Liu et al., 2000), which was also supported by Plötner' s 12S rRNA studies (2001). Cytochrome *b* gene gives information about old and recent events, showing considerable intraspecific variability (Liu et al., 2000). Also, according to comparative genetic distance results of cyt-*b*, Johns and Avise (1998) showed that herpetofauna shows substantially larger genetic distance than mammals and birds. The control region evolves rapidly and informative for recent divergence. It shows extreme variability within populations, among populations, and between ingroup and outgroup taxa (Liu et al., 2000). Further, synonymous mutation rate is 22-fold higher in mtDNA than in nuclear genes. Even the most conserved

tRNA genes evolve 100 times faster than their nuclear counterparts (Saccone et al., 1999).

Consequently, mtDNA is an ideal molecule to (i) trace the maternal ancestry of a population without the confounding effects of biparental inheritance and recombination in nuclear DNA, (ii) analyze ancient DNA and for certain forensic applications (Pakendorf and Stoneking, 2005), (iii) study clonally reproducing taxa, gene flow, dynamics of limited hybrid zones because infraspecific hybridization does not affect the phylogenetic tree construction of mitochondrial DNA sequences (Moritz et al., 1987; Masta et al., 2002), (iv) determine species boundaries (Dawood et al., 2002), (v) clarify molecular inter- and intraspecific relationship among species (Sumida et al., 2002), (vi) investigate different biogeographic hypotheses related to the genetic divergence or species diversity (Symula et al., 2003), and (vii) interpret evolutionary history with respect to paleogeographic events (different biogeographic scenarios) (Fromhage et al., 2004).

The NADH dehydrogenase complex (or mitochondrial respiratory complex) catalyzes the oxidation of NADH by ubiquinone. Then, this is followed by proton transfer across mitochondrial membranes, which is used for ATP synthesis. The complex is composed of many subunits, and some of them are encoded by mtDNA. These genes are ND1, ND2, ND3, ND4, ND4L, ND5, and ND6 (Nosek and Fukuhara, 1994). ND3 gene is 340 bp in length. The transition ratio is 51.4 % and the transversion ratio is 45.9 % between *Rana* and *Xenopus* (Sumida et al., 2001). According to Plötner et al. (2001), in western Palearctic water frogs, the genetic variability of the mt ND3 gene is three times greater than the mt 12S rRNA. The mt ND3 gene has 138 (40.6 %) variable sites whereas mt 12S rRNA region possesses 41 (10.8 %) variable sites. In addition, conspecific populations do not have more than seven substitutions in their mt ND3 gene, and more than ten substitutions usually specify the interspecific level in western Palearctic water frogs. In the same study, using ND3 marker, the relationships among Western Palearctic water frogs were better clarified, and it was suggested that excluding the very close *R. saharica* and *R. perezi* species, there are two water frog clades. One includes *R. lessonae*, *R. bergeri*, *R.*

shqiperica, while the second consists of *R. ridibunda*, *R. bedriagae*, the Asian lake frog group, the Cypriote water frogs, *R. cretensis* and *R. epeirotica*. Furthermore, genetic results show that Anatolian lake frogs do not belong to either *R. bedriagae* or *R. ridibunda*, but are separate undescribed species.

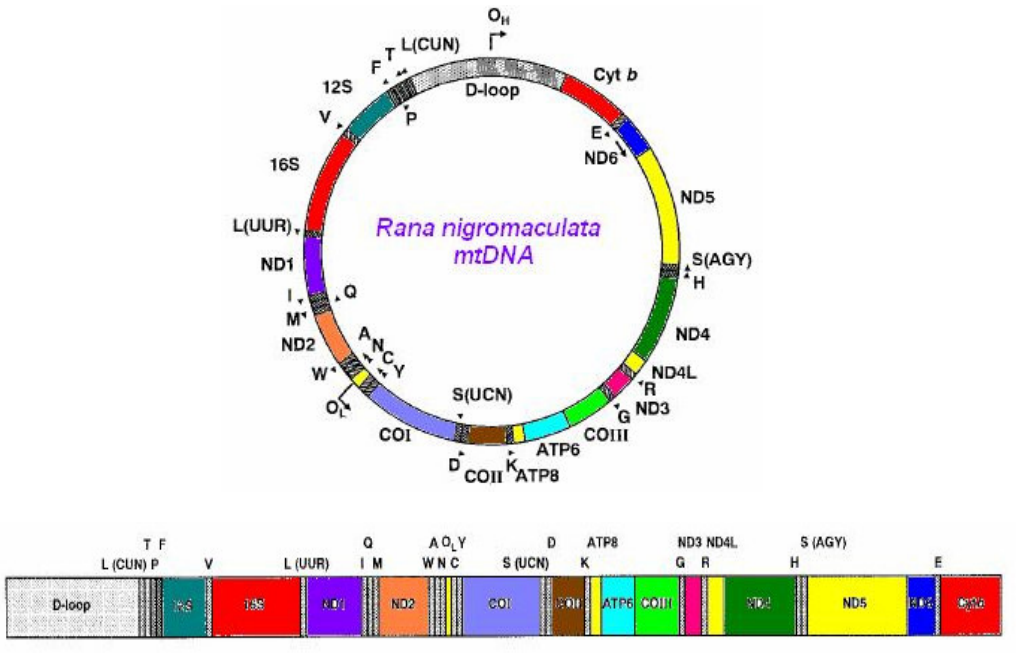


Figure 1-6. Mitochondrial genome organization of *Rana nigromaculata* (Sumida et al., 2001).

1.5. Aim and Scope of the Study:

The aim of the present study was to gain detailed information about *Rana (ridibunda) caralitana* taxon in the southwestern part of Turkey at the molecular level using mitochondrial ND3 marker, in order to:

1- detect the distributional boundaries in *Rana (ridibunda) caralitana* taxon in southwestern Turkey;
2- clarify the taxonomic status of *caralitana* and other water frog forms;
3- show haplotype distribution of mitochondrial ND3 marker in the study area to reveal the genetic structure of different water frog forms;
4- see how well mt ND3 based molecular and morphological results agree;
5- reveal possible hybrid zones and introgression, and
6- better understand the evolutionary relationships between different water frog forms in Turkey.

CHAPTER 2

MATERIAL AND METHODS

2.1. Field Studies

2.1.1. Study Area

The study area consists of areas that surround Beyşehir Lake in Konya (terra typica of *Rana ridibunda caralitana*, Arıkan 1988), occurring within three different ecoregions; Mediterranean Climatic Region, Mediterranean Transitional Region and Continental Region with a distinct climate, vegetation type and topographical features. The Mediterranean Climatic Region has a Mediterranean climate with hot and arid summers, and mild and rainy winters; and is covered by shrubland (*Myrtus spp., Arbutus spp.*) and coniferous forest (*Pinus brutia, Cedrus libani*), whereas Continental Region is characterized by semi-arid continental climate, with cold and wet winters and hot and dry summers, and is covered mainly by steppe vegetation (*Artemissia spp.*, *Festuca spp.*) or dry woodland (*Pinus nigra*, *Quercus spp.*, *Juniperus spp.*). The Mediterranean Transitional Zone shows climatic features between Mediterranean and Continental climates with an intermediate vegetation cover (Atalay, 2002). The altitude changes from sea level to over 2000 m.

During 2004 - 2005, a total of 195 toe samples were collected from 31 different populations (Figure 2-1), from the provinces Konya, Karaman, Burdur, Antalya, Mersin, Adana, Muğla, Denizli, Uşak, Eskişehir, Afyon, Aksaray, Ankara, Edirne, and Artvin (Table 2-1). Sampling was mainly carried out along three transects radiating from Lake Beyşehir toward east, northwest, and south

respectively. Additional samples were obtained as preliminary results necessitated.

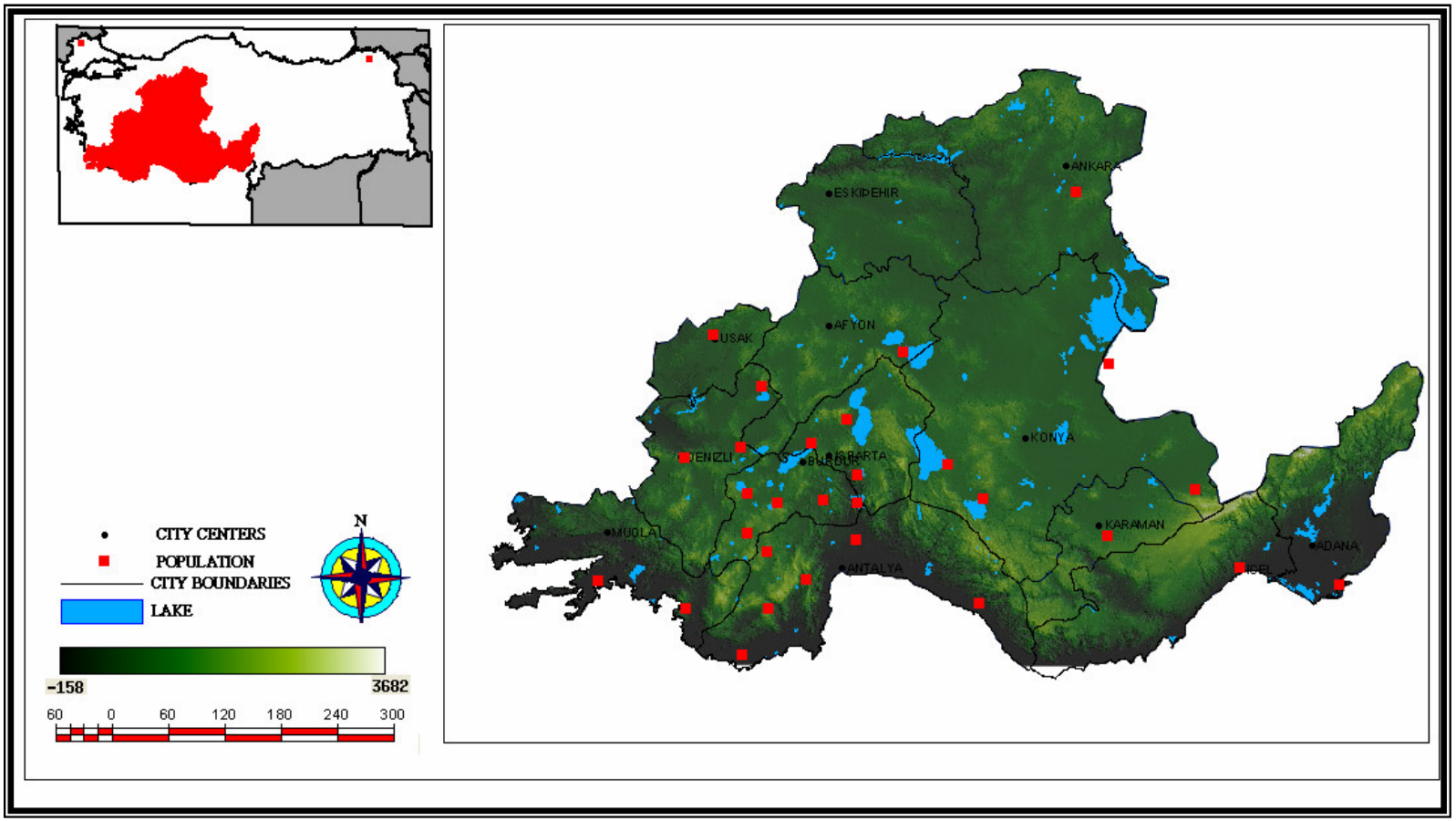


Figure 2-1. Map showing sampling populations in the study area (on the right) and out of area (in the left) by red squares (Kaya, 2006).

Table 2-1. Populations, number of samples taken from each population, their geographic coordinates and river basins on the Southwestern part of Turkey. ("P. color" denotes color codes used in maps for each population).

P.color	Populations	Sample Size	GPS ID	East	North	River Basin
	Yumurtalık	12	gps1	35 36 01.4	36 40 50.7	1
	Mersin	4	gps2	34 37 11.1	36 49 59.9	1
	İvriz	4	gps3	34 10 13.2	37 26 34.5	13
	Gödet Dam	4	gps4	33 18 06.7	37 06 02.4	13
	Eşmekaya	7	gps5	33 18 21.7	38 24 52.2	12
	Ankara	3	gps6	32 57 43.0	39 44 12.0	12
	Akşehir-Eber Lakes	8	gps7	31 16 18.1	38 32 25.8	12
	Seydişehir	4	gps8	31 85 97.2	37 45 00.0	13
	Beyşehir	7	gps9	31 43 36.7	37 40 53.6	13
	Eğirdir Lake	4	gps10	30 43 42.4	38 02 04.1	11
	Burdur Lake	3	gps11	30 38 45.4	37 83 60.5	10
	Sorgun Dam	3	gps12	29 24 19.3	38 42 51.7	6
	Işıklı Lake	6	gps13	29 52 41.5	38 18 39.7	6
	Gemiş	10	gps14	30 16 71.4	37 77 42.7	6
	Gökpınar Dam	4	gps15	29 07 30.7	37 45 79.0	6
	Fethiye	4	gps16	29 08 35.5	36 37 24.2	5
	Kaş	4	gps17	29 42 05.1	36 15 32.1	5
	Marmaris	5	gps18	28 20 10.8	36 50 45.6	5
	Alanya	3	gps19	32 01 79.5	36 35 80.6	3
	Avlan Lake	5	gps20	29 58 09.6	36 36 05.6	5
	Gözlemevi	3	gps21	30 20 06.7	36 49 25.4	4
	Antalya	18	gps22	32 02 43.3	36 36 17.5	4
	Korkuteli	7	gps23	29 56 38.0	37 02 19.4	8
	Tefenni	9	gps24	29 45 11.1	37 11 10.7	7
	Kemer	7	gps25	30 03 11.3	37 25 00.7	10
	Yeşilova	11	gps26	29 44 46.0	37 29 39.8	10
	Bucak	10	gps27	30 30 00.7	37 25 33.5	9
	Karacörenler	15	gps28	30 50 16.1	37 24 02.2	11
	Kovada	10	gps29	30 50 06.0	37 36 31.7	11
	Edirne	5	gps30	41 78 27.6	41 36 26.6	out of area
	Artvin	4	gps31	26 82 51.4	41 69 05.2	out of area

2.1.2. Collection of Specimens

Samples were collected from different types of habitats such as streams, rivers, lakes, ponds, reservoirs, marshy areas and irrigation canals, caught by a fishing rood, dip net or with hands. Firstly, in order to prevent their escape or stop their movement, they were etherized for one minute. Although morphometric parameters were not used in this study, for future research, body, femur, tibia, foot, first toe, and tubercule lengths were measured and recorded (Figure 2-2). Besides, the ventral color of a each specimen was recorded and its photo was taken.

Figure 2-2. Taking external morphometric measurements (Isparta - Kovada, 25.08.2004; Senem Tuğ).

Secondly, for molecular investigations, toe-clipping was carried from each specimen (Figure 2-3). To prevent the infection the wound was disinfected with 80% ethanol. Then, each of toe samples was stored in 80% ethanol at - 20 ^{o}C. According to Berger (1999, 2001), the wounds heal within a few days and toe clipping has no serious effect on survival of metamorphs, the fitness of adults. Furthermore, this method is not expensive and can be applied easily.

Lastly, after measurements and toe-clipping were completed, specimens were released back in their natural habitats. In each locality, geographic coordinates were recorded to construct maps, was drawn using TNT software (Kaya, 2006).

Figure 2-3. Toe-clipping (Burdur Lake, 25.08.2004; Senem Tuğ).

2.2. Morphological Analyses

To determine the extent of morphological variation and correlation between morphological and molecular parameters, a single qualitative parameter, the ventral color of specimens, was used. The presence of orange colored maculations on the ventral part of frogs is a typical feature of the *caralitana* form. Therefore, the geographic range of *caralitana* specific orange color maculation was defined and was tested whether there was a significant correlation between the color and the altitude. The distribution of orange color was finally compared with *caralitana* specific mtDNA haplotypes to identify the geographic areas where these two parameters are approximately concordant. For this purpose, all samples were split into 3 groups according to ventral color maculation:

Group 1: Consists of individuals whose ventral color is either white without spots coded as 1 or white with black-brown spots, coded as 2 (Figure 2-4 / Figure 2-5).

Figure 2-4. White without spots=1 (Adana - Yumurtalık, 09.05.2005; Çiğdem Akın).

Figure 2-5. White with black-brown spots=2 (Denizli - Gökpınar Dam, 24.07.2005; Çiğdem Akın).

Group 2: Includes individuals having orange colored spots on their ventral part, coded as 3 (Figure 2-6).

Figure 2-6. White with orange spots=3 (Konya - Beyşehir,22.09.2005; Çiğdem Akın).

Group 3: Has individuals having orange-brown colored spots, coded as 4 (Figure 2-7).

Figure 2-7. White with orange-brown color spots=4.(Barla - Eğirdir, 24.08.2004, Çiğdem Akın).

2.3. Molecular Studies

2.3.1. Genomic DNA Isolation

The Urea Extraction Procedure (Bilgin et al., unpublished) was used for the isolation of the genomic DNA from toe samples. Nearly 0.01 g of toe samples was resuspended in 750 µL of Urea Extraction Buffer (Table 2-2) in a 1.5 mL eppendorf tube. After addition of 4 µL of 20 mg/mL of proteinase K, the solution was mixed vigorously and incubated at 65 °C for four hours. Then, the equal volume (750 µL) of phenol:chloroform (1:1) was added. The phases were separated by spinning at 13.200 rpm for 10 minutes. The organic phase (bottom) was removed and phenol:chloroform step was repeated. 300 µL of the aqueous phase was recovered and transferred carefully into a fresh tube. 0,7-volume of isopropanol was added into each sample and mixed carefully, centrifuged at 13.200 rpm for 5 minutes. After the removal of the supernatant, 1000 µL of

absolute ethanol was added, and the pellets were washed by inverting the tubes several times. Tubes were kept on ice for 10 minutes, and spinned at 13.200 rpm for 5 minutes. After the ethanol was discarded gently without dropping the pellet, the crude DNA was air dried for 5 minutes at room temperature. DNA pellets were resuspended in 500 µL of TE-Acetate-RNase solution (Table 2-2), and incubated at 37 °C for o/n. 2 volume of absolute ethanol and 0.1 volume of 3M sodium acetate pH:5.2 were added into each tube and mixed. Tubes were stored at – 20 °C for o/n. The next day, they were centrifuged at 13.200 rpm for 10 minutes. Supernatants were again removed carefully without distrupting the pellet of DNA. 800 µL of 70 % ethanol was added and spinned at 13.200 rpm for 10 minutes. After pouring the ethanol out, the tubes were air dried at room temperature for 5 minutes until all ethanol was removed. Then, the DNA was resuspended in 100 µL of TE buffer (Table 2-2) pH: 8.0 and stored at -20 °C for long-term storage. Concentration of genomic DNA was checked both by performing agarose gel electrophoresis and spectrophotometrically. To determine the concentration and purity of DNA, it was diluted and its optical density (OD) was read at 260 nm and 280 nm. Samples having an OD_{260} /OD_{280} ratio < 1.8 were be re-extracted with phenol:chloroform to remove the protein contamination. To calculate the concentration of DNA, the equation used was "concentration (µg/mL) = A_{260} x 50 x dilution factor ".

2.3.2. Agarose Gel Electrophoresis

Electrophoresis was carried out a horizontal electrophoresis apparatus. 0.7 % agarose gel was prepared in TAE buffer (Table 2-2) and ethidium bromide was added to give a 0.5 µg/mL final concentration in melted agarose gel. Electrophoresis was performed at 80 Volts for 45 minutes. The DNA bands were visualized on a softwave UV transilluminators (UVP) and photographed by using Vilber Laurmat Gel Imaging System. Lambda *Pst*I DNA marker was used to determine the length of DNA bands (Figure 2-8).

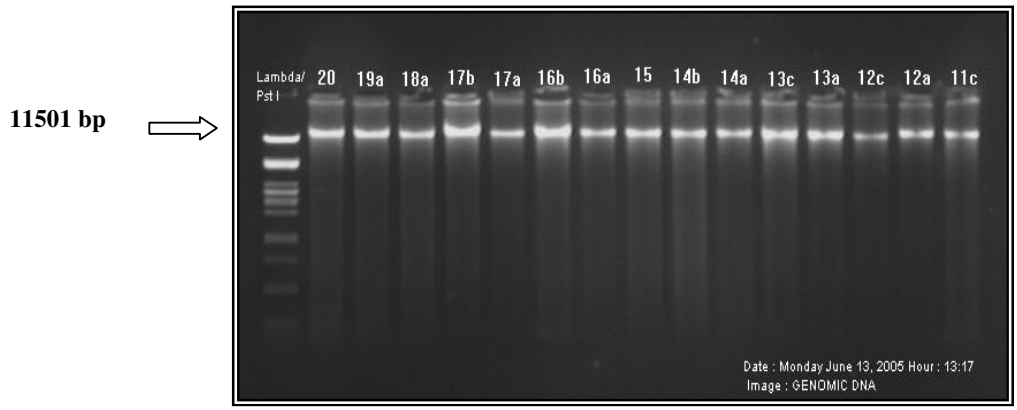


Figure 2-8. Extracted total DNA from individuals by using Urea Extraction Procedure. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2-15: Extracted genomic DNA bands.

Table 2-2. preparation of buffers.

UEB (Urea Extraction Buffer):

3 M Urea

20 mM EDTA

1% N-Lauryl-sarcosine-Na-salt

50 mM Tris buffer (pH=8.0)

125 mM Na_2SO_4

1% PVP (10000mw)

PHENOL:CHLOROFORM:

Mix one volume of phenol (Tris buffer equilibrated; pH=7.9) and one volume of chloroform.

Table 2-2. continued.

TE Buffer:

10 mM Tris buffer

1 mM EDTA

pH is set to 8.0 and sterilized at 121 °C for 15 minutes.

TAE Buffer 50X (for agarose gel):

Tris base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH:8.0)	100 mL
Distilled water	350 mL

Tris Stock Solution (1M):

Tris base	121,14 g
Distilled water	350 mL

pH is adjusted to 8.0 by HCl, distilled water to 500 mL, and stored at 4 °C.

EDTA Stock Solution (0.5M):

EDTA	380,2 g
Distilled water	200 mL

pH is adjusted to 8.0 by 3M of NaOH, distilled water to 250 mL, and stored at 4 °C.

NH_4OAc Stock Solution (5M):

NH_4OAc	96,35 g

Distilled water to 250 mL.

TE-Acetate-RNase Buffer:

50 mM Tris buffer pH=8

10 mM EDTA

0.5 NH_4OAc (5 M stock)

20 µg/mL RNase A (100 mg/mL stock

2.3.3. Primer Design for Mitochondrial ND3 Gene

For amplification of ND3 gene, the primers were designed on the basis of nucleotide sequence of the *Xenopus laevis* mtDNA (the Gene Bank accession number M10217) at the Institute of Systematic Zoology in Berlin (Ohst, 1999). ND3 gene on water frogs corresponds to the position 11562-11904 (ND3) in the mt genome of *Xenopus laevis* (Roe et al., 1985). The regions for the primers designed are shown in Figure 2-9. The nucleotide sequences of the primers are shown in Table 2-3. The primers were synthesized commercially by Iontek (İstanbul, Turkey) and Genmed (Ankara, Turkey).

Figure 2-9. Nucleotide sequence of ND3 gene of *Xenopus laevis*. The lines show regions where the primers bind.
(http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=343717).

```
                                                          ND3F
(11562).....................................................5'agtacacgtga
cttccaatca caaagtctta gttagaatct aagagaaagt aatgacagcc actatcctaa
taattgccat aactctatca actattctag caatcttaag tttttgactt ccccaaataa
cccctgatat agaaaaactc tccccctacg agtgtggatt tgatcctctg ggctctatgc
gattaccatt ctccatacga ttcttcttga tcgccatttt atttcttcta tttgacctag
agattgcgct tcttctccct ttcccttgag ccgcacaact taacacacca agtattgtaa
tcttatgagc agctctaatt ctaacccttc ttactcttgg cctaatttat gaatgacttc
aaggaggcct agaatgagct gaatgagttg ttagtctaaa caagacagtt gatttcggct
caa 3'.................................(11904)        ND3R
```

Table 2-3. ND3 primers used in PCR amplification.

Primer name	Nucleotide Sequence	Size of the PCR products (bp)
ND3F	5' AGTACACGTGACTTCCAATC 3'	340
ND3R	5' TTGAGCCGAAATCAACTGTC 3'	340

2.3.4. Polymerase Chain Reaction for ND3 Gene

PCR reaction mixture contained 5 µL of 10 x PCR buffer, 4 µL of 25 mM $MgCI_2$, 20 pmols of each primers, 1 µL of 10 mM dNTP, 0.5 unit of JMR Super Therm DNA polymerase, 0.1 µg genomic DNA and 33.5 µL of dH_2O to complete the volume to 50 µL. PCR for the amplification of ND3 gene was carried out according to Plötner et al. (2001). After the initial denaturation step of 5 minutes at 94 °C, samples were subjected to 50 cycles consisting of 30 seconds at 96 °C, 30 second at 50 °C and 1.30 seconds at 74 °C. The final extension was performed at 74 °C for 7 minutes. Reaction mixtures were run in a 1.4 % agarose gel for 1.5 hours at 90 Volts (Figure 2-10). PCR products were extracted from the gel as mentioned in Section 2.3.2.5.

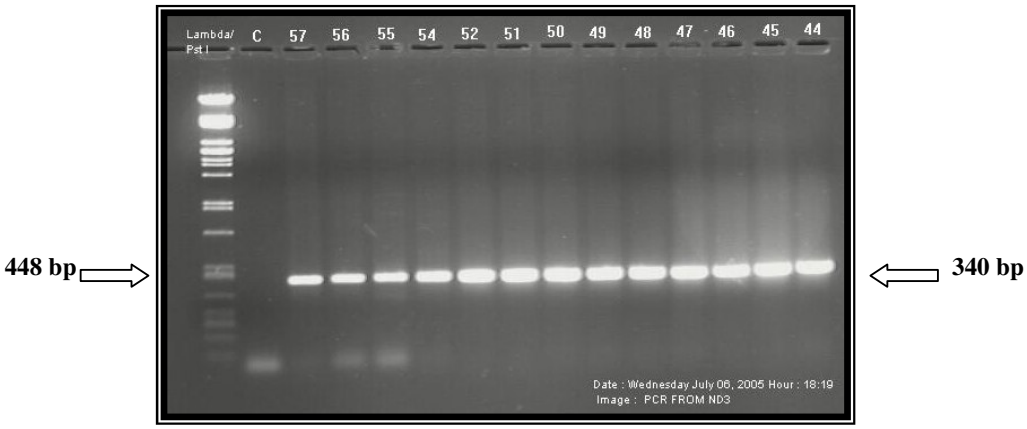


Figure 2-10. Amplification of mitochondrial ND3 gene from the frog genomic DNA. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2: negative control (no template), Lane 3-15: 340 bp PCR product obtained with the primers ND3F and ND3R.

2.3.5. Extraction of PCR Products from Agarose Gel

After separation of PCR products in agarose gel, desired fragments were extracted from the gel by using the Gel Extraction Kit (Qiagen Inc., Valencia, CA; Gene Mark). The PCR band containing amplified region was excised from the gel and weighed. DNA in this gel slice was recovered according to Qiagen's instructions. Then, a recovered aliquot was run on an agarose gel to asses the molecular weight (Figure 2-11).

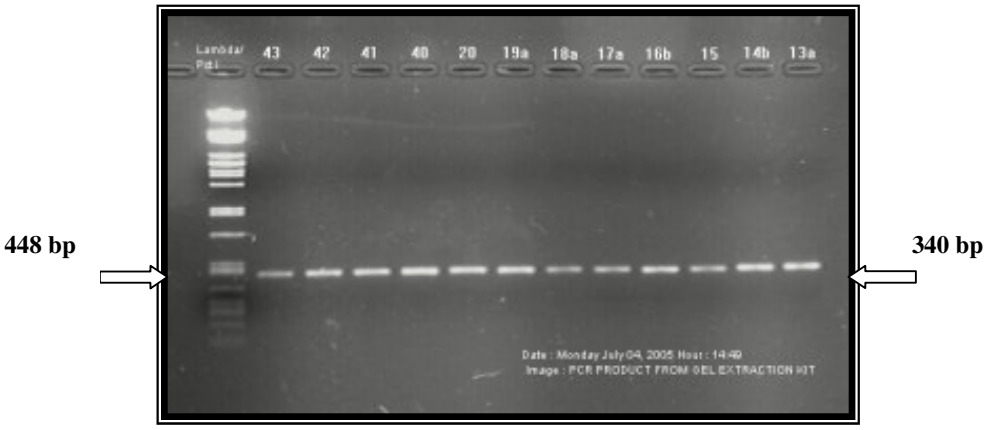


Figure 2-11. Extracted ND3 PCR fragments from agarose gel. Lane 1: Marker (*Pst*I digested Lambda DNA), Lane 2 -12: 340 bp PCR products cleaned by the kit.

2.3.6. Sequencing Reactions

For ND3 gene for mtDNA, DNA sequencing was carried out at İontek Inc. Laboratories (İstanbul, Turkey) by using DYEnamic ET Terminator Cycle

Sequencing Kit (Amersham). Sequencing analysis (electrophoresis and detection of the fluorescent dye labeled nucleotide fragments) was performed with an automatic DNA sequencer (ABI Prism 310 Genetic Analyzer). All PCR fragments were sequenced with light strand primer (Figure 2-12). Sequences were aligned using the CLUSTAL W algorithm tool of MEGA version 3.1 (Kumar et al., 2004) (Figure 2-13). Deduced nucleotide sequence data was compared with the National Center for Biotechnology Information (NCBI) database using the BLAST search at the web site (http://www.ncbi.nlm.nih.gov/BLAST/) to find out mtDNA haplotypes.

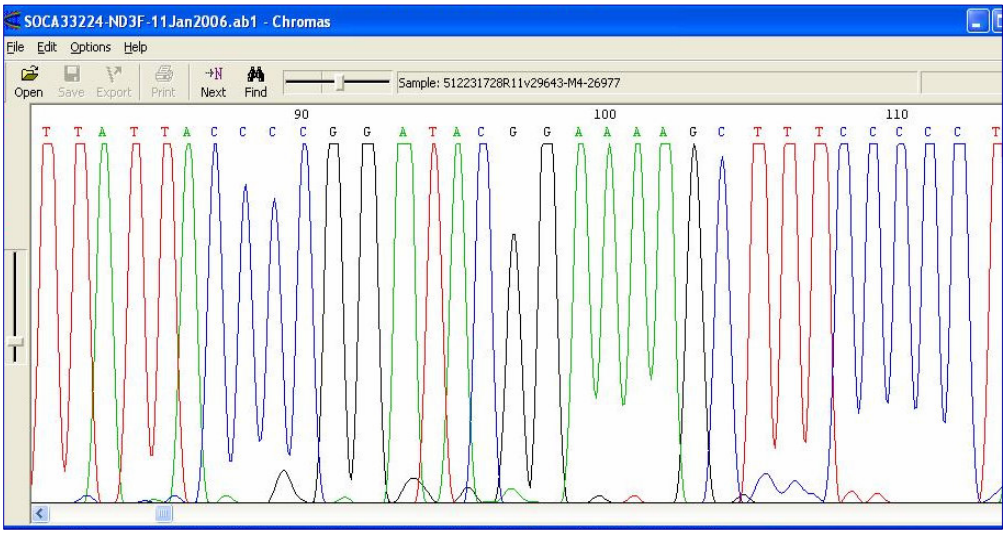
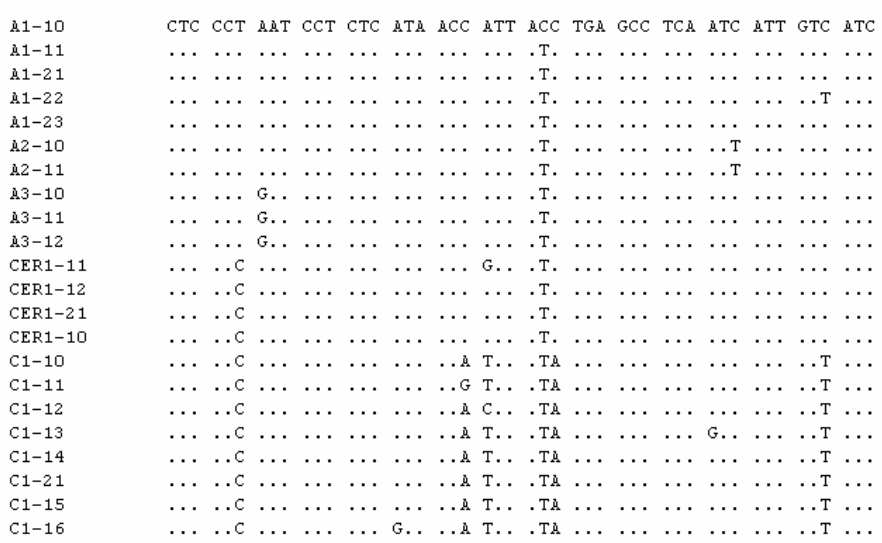


Figure 2-12. A chromatogram file from DNA sequencing reaction using the light strand primer.



A1-10	CTC	CCT	AAT	CCT	CTC	ATA	ACC	ATT	ACC	TGA	GCC	TCA	ATC	ATT	GTC	ATC
A1-11	...	...	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...
A1-21	...	...	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...
A1-22	...	...	...	...	...	...	...	...	.T.	...	...	...	...	...	..T	...
A1-23	...	...	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...
A2-10	...	...	...	...	...	...	...	...	.T.	...	...	...	..T	...	...	...
A2-11	...	...	...	...	...	...	...	...	.T.	...	...	...	..T	...	...	...
A3-10	...	...	G..	...	...	...	...	...	.T.	...	...	...	...	...	...	...
A3-11	...	...	G..	...	...	...	...	...	.T.	...	...	...	...	...	...	...
A3-12	...	...	G..	...	...	...	...	...	.T.	...	...	...	...	...	...	...
CER1-11	...	..C	...	...	...	...	...	G..	.T.	...	...	...	...	...	...	...
CER1-12	...	..C	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...
CER1-21	...	..C	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...
CER1-10	...	..C	...	...	...	...	...	...	.T.	...	...	...	...	...	...	...
C1-10	...	..C	...	...	...	...	...	..A	T..	.TA	...	...	...	...	..T	...
C1-11	...	..C	...	...	...	...	...	..G	T..	.TA	...	...	...	...	..T	...
C1-12	...	..C	...	...	...	...	...	..A	C..	.TA	...	...	...	...	..T	...
C1-13	...	..C	...	...	...	...	...	..A	T..	.TA	...	...	...	G..	...	..T	...
C1-14	...	..C	...	...	...	...	...	..A	T..	.TA	...	...	...	...	...	..T	...
C1-21	...	..C	...	...	...	...	...	..A	T..	.TA	...	...	...	...	...	..T	...
C1-15	...	..C	...	...	...	...	...	..A	T..	.TA	...	...	...	...	...	..T	...
C1-16	...	..C	...	...	...	G..	...	..A	T..	.TA	...	...	...	...	...	..T	...

Figure 2-13. Sequence alignment obtained from different haplotypes showing the positions of transitions and transversions in the ND3 gene.

2.4. Molecular Data Analyses

2.4.1. Basic Statistical Analyses

2.4.1.1. Mitochondrial DNA Haplotypes

Each of haplotypes identified was grouped into the main haplogroups (MHG). A MHG consists of a group of mtDNA haplotypes that can be used to define the geographically oriented genetic populations, and has specific substitutions and a clear divergence of the remaining homologous sequences of a monophyletic group (Plötner and Ohst, 2001). Additionally, 7 of mtDNA sequences from the gene bank with accession numbers: AJ313135 (Adana-Ceyhan), AJ310337 (Ankara), AJ310316, AJ313132, AJ313133 (Konya-Beyşehir), AJ313131 (Muğla-

Akçapınar), AJ310314 (Antalya-Alanya) and one sequence from J. Plötner's database (Systematic Zoology Institute, Museum fur Naturkunde (Berlin, Germany) with the number Hotz17207 (Karpathos) were combined to our sequence results. The rate of divergence between two sequences can be calculated by the Jukes and Cantor substitution model (1969). For our data set, the Jukes and Cantor distance is equal to or greater than 0,018 with at least 6 substitutions and several specific characters in sequences used as criteria to attribute different MHGs. 0,018 is the smallest value between two distinct Anatolian clades.

2.4.1.2. Measuring Genetic Variation in DNA Sequences

In order to define the extent of DNA polymorphism, three common measures were computed by the Arlequin software version 3.01. (Excoffier et al., 2005).

2.4.1.2.1. Haplotype diversity

It is the probability that two randomly chosen haplotypes are different in the sample, computed as

$$\hat{H} = \frac{n}{n-1}\left(1 - \sum_{i=1}^{k} p_i^2\right)$$

where n is the number of gene copies in the sample, k is the number of haplotypes and p_i is the sample frequency of the i^{th} haplotype (Nei, 1987 referred in Excoffier et al., 2005).

2.4.1.2.2. Number of segregating sites

Any nucleotide site that represents two or more nucleotides among all aligned sequences is called as segregating or polymorphic site (Nei and Kumar, 2000). The basic parameter of genetic variation (θ) is estimated using S:

$$\theta = \frac{S}{a_1}$$

where $a_1 = \sum_{i=1}^{n-1} \frac{1}{i}$. (Tajima, 1989)

Nevertheless, this measure depends on the length of sequence analyzed and the number of samples. Therefore, the longer and more sequences are studied, the more segregating sites can be found (Jobling et al., 2004).

2.4.1.2.3. Nucleotide diversity

It is the average number of nucleotide differences per site between two randomly chosen DNA sequences in the sample (Nei and Li, 1979). In contrast to segregating site, this DNA polymorphism measure does not depend on the sample size (Nei and Kumar, 2000).

In order to estimate mean number of differences between all pairs of haplotypes, the following equation was used (Tajima 1983):

$$\hat{\pi} = \sum_{i=1}^{k} \sum_{j=1}^{k} p_i p_j \hat{d}_{ij}$$

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**.

2.4.1.3. Estimation of Nucleotide Substitutions for mt ND3 Gene: Jukes and Cantor's Method

To estimate nucleotide substitutions, many different distance models from a simple to sophisticated were developed. Jukes and Cantor method is one of the simple model for calculation of distances among nucleotide sequences (Jukes and Cantor, 1969). This model suggests that change of one nucleotide to another at one site has equal frequency with any substitution among other pairs. In other words, nucleotide substitution rate is the same for all pair of nucleotides A, G, C, T. Actually, transition occurs more frequently than transversion, which is the main assumption for Kimura 2 Parameter model. However, in this model, there is no transition and transversion bias. All types of substitution were accepted as *α*. Furthermore, it was assumed that all bases are present in equal frequency at the nucleotide sequences, and similarly the rate of nucleotide substitution among sites is equal to each other.

Number of nucleotide substitution per site *(d)* is calculated using the formula:

$$d = -(3/4)\ln[1-(4/3)p]$$

p: proportion of different nucleotide between sequences.

	A	G	C	T
A	-	α	α	α
G	α	-	α	α
C	α	α	-	α
T	α	α	α	-

Figure 2-14. Jukes and Cantor method

In this study, to study closely related sequences, Jukes and Cantor approach, a simple model is preferred, because the Tamura, Kimura, Tamura - Nei and Jukes and Cantor distances are identical to each other when d value is equal or smaller than 0,25. Unless transition/transversion ratio *(R)* is high *(R>5)* and number of nucleotides examined *(n)* is very large, Jukes and Cantor is better, having a smaller variance compared to more complex approaches. In addition, for constructing phylogenetic tree from distance measures, simple one is as effective as more advance distances for obtaining correct topology under certain circumstances (Tajima and Takezaki, 1994; Nei and Kumar, 2000).

2.4.2. Phylogenetic Analyses

2.4.2.1. Phylogenetic Tree Construction Based on a Distance Method: Neighbour Joining (NJ) Method

NJ tree is based on minimum evolution, developed by Saitou and Nei (1987). Principally, the method starts to find pairs of OTU (neighbor) having minimum length with a starlike tree. Then each clustering step of OTUs, the total branch length is reduced. Therefore, the branch of final tree is smaller than the starlike tree. To define true neighbors, the sum of branch lengths among different taxa are calculated. For this reason, using equation, a distance matrix is constructed. Taxa with the smallest distance are chosen and connected by a single node. Later, the distance between this node and the remaining taxa was recalculated and again the smallest neighbors are connected. This procedure is repeated until all taxa are clustered in a single unrooted tree.

A major advantage of this method is that the topology of tree and the branch length can be quickly achieved. Namely, it is quiet efficient and fast for large number of datasets even with a personal computer and suited for bootstrap analysis. However, NJ method does not include backward and parallel

substitution, hence; underestimates the homoplasmy and long evolutionary distances and reduces information. Furthermore, it produces a single unrooted tree. Therefore, it is not easy to obtain a true tree wihout some statistical test to understand the reliability of a constructed tree (Nei and Kumar, 2000).

2.4.2.2. Testing Accuracies of Phylogenetic Trees: Bootstrapping

After a phylogenetic tree is obtained, it is necessary to evaluate the reliability of the constructed tree because of topological errors and branch length errors. There are 2 types of statistical methods to test the branching pattern of phylogenetic trees. One of them is the bootstrap test, first developed by Felsenstein (1985). The other method is the interior-branch test, which examine whether or not the internal branch length is significantly positive in a given tree topology (Nei et al., 1985; Dopazo 1994; Sitnikova et al., 1995; 1996).

The bootstrap test is based on a reassambling technique (Efron, 1982). Basically, *n* nucleotide sites are chosen randomly with replacement from the dataset to construct another phylogenetic tree. Meanwhile, some sites may be selected many times, others may not be chosen. This tree topology is compared with the original tree. Any internal branch of the original tree is the same as that of the bootstrap tree is taken 1 identity value, whereas others are taken 0. This procedure is repeated many times, and all 1 identity values of original tree are calculated as a percentage, called as bootstrap value. Principally, if the value is greater or equal to 95 %, the accuracy of the original tree is accepted as statistically significant. In NJ tree, applications of the bootstrap test is quite quick and efficient and the interpretation of results is easy. Particularly, if the numbers of sequences and nucleotides are great, each part of DNA sequence evolved independently, the distances measure for the number of nucleotide substitution is an unbiased, the internal branch length is 0, which is the null hypothesis for the bootstrap test. If a phylogenetic tree has low bootstrap values

for internal branch length, the application of the condensed tree (a multifurcating tree) is useful, which reduces the internal branch length to 0 with bootstrap values of 50 % or less. Therefore, this tree makes clear the reliable portions of the original tree, while eliminates interior branches with a low importance (Nei and Kumar, 2000).

2.4.2.3. Minimum Spanning Network (MSN)

At the population level, especially dealing with closely related sequences, network methods have advantages over the traditional phylogenetic methods such as representing the persistence of ancestral haplotypes and multifurcation to many descendant haplotypes in the population, showing reticulate relationships within population due to recombination between genes, hybridization between lineages and homoplasy (Posada and Crandall, 2001; Cassens et al., 2005). MSN has been developed under molecular variance parsimony technique to include all possible MSTs within a single network (Excoffier and Smouse, 1994). In the molecular variance parsimony technique, each competing MST is translated into a matrix of Patristic Distances among haplotypes to calculate a set of population statistics: function of haplotype frequencies, squared patristic distances among haplotypes and geographic partitioning of populations.

The Arlequin software version 3.01 (was used to implement the computation of a Minimum Spanning Network between OTUs (Operational Taxonomic Units) based on the matrix of pairwise distances calculated between all pairs of haplotypes. A MSN was constructed by hand, by following the Jobling et al. (2004) procedure:

- a character-based distance matrix between all haplotypes is computed,
- links are drawn between all haplotypes separated by single mutational steps,

- mutational steps of increasing size are taken into consideration until all haplotypes are linked a single network and all most parsimonious links from any haplotypes have been reconstructed.

2.4.3. Identification of the Genetic Structure of Groups of Populations: SAMOVA

Spatial analysis of molecular variance (SAMOVA) was developed for defining groups of populations that are geographically homogeneous and genetically maximally differentiated from each other; hence, it helps to detect zones of sharp genetic changes in a geographical area (Dupanloup et al., 2002). The method is based on a simulated annealing procedure to maximize the proportion of total genetic variance due to differences between groups of population. The simulated annealing approach prevents becoming trapped at a local optimum, and as times passes, it should get closer to a global optimum and become less prone to departure from that optimum.

The SAMOVA 1.0 software was used to geographic regions of large genetic changes among populations. Initially, n sampled populations were randomly partitioned into K groups. Then, the simulating annealing procedure was used to find the composition of the K groups and to maximize the F_{CT} index, which is the total genetic variance due to differences between groups of populations. SAMOVA was run using 100 simulated annealing processes for K= 2 to 9. The highest F_{CT} value achieved from these runs was retained as the best population grouping scheme, as suggested by Dupanloup et al. (2002).

2.4.4. Testing of Population Subdivisions: Analysis of Molecular Variance (AMOVA)

AMOVA was developed to estimate haplotype diversity at different hierarchical levels such as among individuals within populations, among populations within groups or among populations (Excoffier et al., 1992). Genetic distance matrices obtained from differences among DNA sequences is converted into molecular variance consisting of different covariance components. Those component are used to estimate *F-statistics* analogs, called as *Φ-statistics* which are haplotypic correlation measures and, similarly includes 3 statistics: Φ_{ST} is the measure of genetic variation within populations, Φ_{SC} is the measure of genetic variation among populations within groups, and Φ_{CT} is the measure of genetic variation among groups.

The test of AMOVA was constructed by the Arlequin software version 3.01. (Excoffier et al., 2005), based on some formulas (Table 2.5). To test the significance of the *Φ-statistics*, 1000 random permutations of different hierarchical levels were performed.

Table 2.4. AMOVA table was taken from Manual Arlequin ver 3.01 (Excoffier et al., 2005).

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

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 gene copies for haplotypic data.

2.4.5. Making Inferences about Historical Demography of Populations

2.4.5.1. Mismatch distribution

Mismatch distribution graphs represent the distribution of the observed number of base substitution differences between pairs of haplotypes. As well as summarizing the overall genetic diversity within a sample, the shape of the distribution can be used as an indicator of population history. This distribution is generally smooth, unimodal, reflecting a period of rapid population growth from a single haplotype (recent demographic expansion), and gene genealogy has a star-like phylogeny with an excess of rare mutations, whereas a ragged, multimodal distribution indicates samples drawn from populations at demographic equilibrium or constant over a long period of time, and gene genealogy presents a mixture of both very short and very long branch lengths (Hudson and Slatkin, 1991; Rogers and Harpending 1992; Harpending and Rogers, 2000; Ray et al., 2003).

Under the infinite sites model (Kimura, 1969), mismatch distribution for different haplogroups was constructed using the software DnaSP version 4.10.9. It was assumed that t generation ago a stationary haploid population at equilibrium has suddenly changed from a population size of N_0 to a new size of

N_1 and it remained at that size ever since. Then, the probability of observing i differences between two haplotypes taken at random from this population, as

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

(Li, 1977), where $\theta_0 = 2uN_0$, $\theta_1 = 2uN_1$, $\tau = 2ut$, N is the population size, t is the generation time, u is the total mutation rate per generation and per sequence. Therefore, this model based on 3 demeographic parameters directly estimated from mismatch distribution. Theta Initial: θ_0 (theta before population growth or decline), theta Final: θ_1 (theta after population growth or decline), Thau (τ) is the date of the growth or decline measured in the units of mutational time (Rogers and Harpending, 1992).

DnaSP computes the observed mismatch distributions and expected values both in a stable population and in growing and declining populations (Hudson and Slatkin, 1991; Rogers and Harpending 1992). Furthermore, it estimates the raggedness statistics for quantifying the smoothness of the observed mismatch distribution and to distinguish data from expanded and stationary population (Harpending, 1994).

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

where d is the maximum number of observed differences between haplotypes, and x' s are the observed relative frequencies of the mismatch classes. This value is small for smoother distributions (equal or smaller than 0,04) observed in expanded populations than multimodal distributions usually found in stationary populations.

However, the raggedness statistic has low statistical power; thus, the neutrality tests were applied to detect the past demography of populations (Ramos-Onsins and Rozas, 2002).

2.4.5.2. Neutrality tests

DnaSP 4.10.9 software was used to compute all the neutrality tests (Rozas et al., 2003) mentioned below.

2.4.5.2.1. Tajima' D Test

Tajima (1989b) developed D test under the infinite site model (Kimura 1969). This test compares the average number of nucleotide differences between sequences randomly drawn from a sample, π, to the number of segregating sites, S. It is calculated as

$$D = \frac{\hat{\theta}_\pi - \hat{\theta}_S}{\sqrt{Var(\hat{\theta}_\pi - \hat{\theta}_S)}},$$

S is strongly affected by the existence of deleterious alleles because deleterious alleles are usually kept in low frequency but S ignores the frequency of mutants. However, π is not so much affected by the presence of deleterious alleles because it considers the frequency of mutants. Therefore, the presence of selective effects on sequences, the estimate of θ based on the number of segregating sites (S) will be different from the estimate based on the number of nucleotide differences (π). Consequently, under the neutral model, in a population of stationary size, $\pi = S$. A negative D value indicates the occurrence of a sudden and large expansion after a bottleneck or small founder event, selective sweep (complete hitchhiking) or background selection of slightly deleterious alleles. A positive value indicates either balancing selection, secondary contact among previously isolated populations and the effect of mutation rate heterogeneity on DNA sequences (Aris-Brosou and Excoffier, 1996; Tajima, 1996; Charlesworth et al., 1993).

2.4.5.2.2. Fu and Li' s D* and F* Tests

These tests were developed under the assumption of selective neutrality (Fu and Li, 1993).

D* test 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.

F* test is based on the differences between η_S, the number of singletons and k, the average number of nucleotide differences between pairs of sequences.

The significant negative value means excess of mutations in external branches due to population expansion, migration, purifying selection, selective sweep, background selection or bottleneck while the positive value indicates mutation deficiency in external branches because of balancing selection, and population subdivision (Fu and Li, 1993; Simonsen et al., 1995).

2.4.5.2.3. Fu's Fs Test

Fu (1997) developed the Fs test based on the haplotype (gene) frequency distribution conditional the value of θ. This test takes negative value when an excess of recent mutation (or rare alleles), which can be an indicator against the neutrality of mutations. It is the most powerful test to detect population growth, particularly for large sample size. If only Fs has significant negative value, it can be due to population growth and genetic hitchhiking. On the other hand, only significant D* and F* values probably represents the background selection (Fu, 1997; Ramos-Onsins and Rozas, 2002).

2.4.6. List of Statistical Software and Their Webpage Addresses

- Chromas version 2.01.

http://www.technelysium.com.au/chromas_lite.html, December 2006.

- MEGA version 3.1 (Molecular Evolutionary Genetics Analysis).

http://www.megasoftware.net/mega.html, December 2006.

- Arlequin version 3.1.

http://cmpg.unibe.ch/software/arlequin3/, Zoologie Institute, University of Berne, December 2006.

- SAMOVA version 1.0 (Spatial Analysis of Molecular Variance).

http://web.unife.it/progetti/genetica/Isabelle/samova.html, Department Of Biology, University of Ferrara, December 2006.

- DnaSP version 4.10.9. DNA Sequence Polymorphism.

http://www.ub.es/dnasp/, University of Barcelona, December 2006.

- TNTlite 2006:72

http://www.microimages.com/tntlite/osdialog.htm, December 2006. All maps were constructed using TNTlite software by Kaya (2006).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Morphological Results

3.1.1. Geographic Variation in Ventral Color

With previous and this study, the geographic range of *caralitana* specific orange colored maculation (coded as 3) was further extended to Eşmekaya - Aksaray on the north, to İvriz - Konya on the east, to Işıklı Lake - Denizli and Tefenni - Burdur on the west, and to Avlan Lake and Düzlerçamı - Antalya on the south (orange colored pie chart in Figure 3-1).

Remarkably, individuals coded as 4 (maroon colored pie chart in Figure 3-1), having orange-brown spots on their ventral part were found in Korkuteli - Antalya, Tefenni, Kemer - Burdur, Gemiş - Denizli, Eşmekaya - Aksaray populations which are the transition regions between orange colored and black-brown colored spotted individuals, and also in Kovada, Suçatı, and Eğirdir - Isparta populations. These specimens show an intermediate color in their ventral spots and could probably hybrids, which is also supported by previous studies (Arıkan, 1990; Arıkan et al., 1994; Jdeidi et al., 2001).

Specimens with a white colored venter (coded as 1) were mainly recorded in the coastal parts of Mediterranean region, especially in Fethiye - Muğla to Yumurtalık - Adana except for some populations from Antalya. However, previous work (Arıkan, 1990; Arıkan et al., 1994) has found white ventral colored individuals together with specimens having black-brown colored spots in

other regions of Turkey; thus, its distribution is not restricted to coastal Mediterranean region.

Except for Lake District populations, specimens with white and black-brown colored maculation (coded as 2) were found substantially widespread in many populations along with white ventered individuals (coded 1) (black-colored pie chart in Figure 3-1).

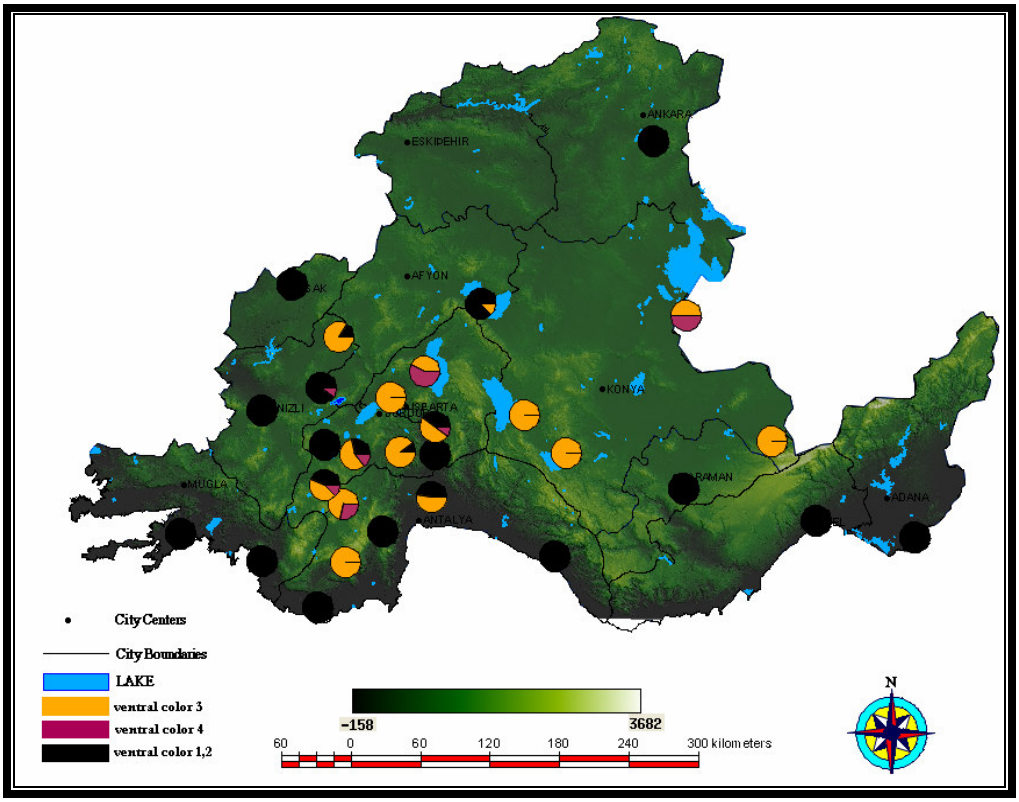


Figure 3-1. Map showing the distribution of ventral colors in the study area. Orange color pie chart: *caralitana* specific orange colored maculation (3); black color pie chart: white colored or white with black colored maculation (1,2); maroon color pie chart: orange-brown colored maculation (4) (Kaya, 2006).

The distribution pattern of ventral color maculation in the study area clearly indicated that ventral color exhibits a complex clinal variation especially in western Lakes District. Furthermore, different colored individuals are seen syntopically in those transition regions. In other regions, there seems to be abrupt changes probably due to geographic barriers such mountains.

Some of the specimens were caught as juveniles; these had a white ventral color on their venters. It is not clear if the ventral color of juveniles attains a darker color at the adult stage. In previous studies, it was suggested that color change is age-related and occurs in one direction (Hoffman and Blouin, 2000; Summers et al., 2003). Thus, the ontogenetic change of orange color from a juvenile morph to into an adult and its mode of inheritance are not known. An orange colored maculation on the venter might be a response to environmental variation or a defense mechanism against predation, which require further investigation.

3.1.2. Effect of Altitude on Ventral Color

To see whether there is any correlation between orange colored maculation and altitude, a linear regression analysis was performed by using the percentage of orange colored maculation as a dependent variable and the altitude as an independent variable (N=56). Results showed that there is no a significant relationship between two parameters with $R^2 = 0{,}0898$ (Figure 3-2).

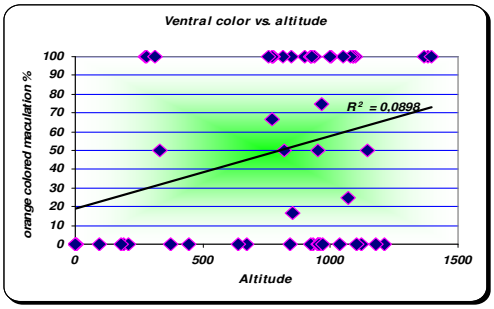


Figure 3-2. Percentage of ventral color versus altitude.

Although Sinsch and Schneider (1999) suggested the presence of a positive correlation between size and altitude, and a continuous size distribution among lowland and highland specimens, our more detailed study did not support this assumption. The proportion of individuals with orange colored spots ranges between 17 to 100% at a wide range of altitudes from 276 m to 1380 m.

3.2. Results of Molecular Analyses

3.2.1. Basic Statistical Results

3.2.1.1. Mitochondrial ND3 Haplogroups / Haplotypes

In this study, a total of 203 mtDNA sequences were investigated. Among the analysed 340 bp of the ND3 gene, 55 variable sites or number of observed sites with substitutions (16.2 %), 9 singelton sites (2.6 %) and 46 parsimoniously informative sites (13.5 %) were identified with the relative values of nucleotide composition, C:T:A:G= 30.79:33.99:19.77:15.46.

In 203 of investigated sequences a total of 39 haplotypes were found with 0.867 of haplotype diversity, 7.784 of mean number of pairwise differences and 7.839 of number of segregating sites values (Table 3.2). According to the lowest value of Jukes and Cantor distance (d_{JK}) (0.018), respective geographic distributions and specific characters in sequences, they were classified into 4 MHGs (mainhaplogroups). These four groups were named either after the accepted taxon they most likely belong to, or in case a new taxon is likely, after the geographical names of their respective ranges: Caralitana MHG, Anatoliaca MHG, Ceyhanensis MGH, and Ridibunda MHG. Furthermore, Caralitana MHG was divided into 3 SHGs (subhaplogroups): Caralitana1 (C1 SHG), Caralitana2 (C2 SHG) and Caralitana5 (C5 SHG), while the Anatoliaca MHG divided into 2 SHGs: Anatoliaca1 (A1 SHG) and Cerigensis1 (Cer1 SHG) since d_{JC} between

SHGs within Caralitana MHG and Anatoliaca MHG has both lower and higher values than 0.018.

MHGs, haplotypes and their populations are presented in Table 3.1. For the samples, abbreviatons of C (Caralitana), A (Anatoliaca), Cey (Ceyhanensis), Cer (Cerigensis) and Rd (Ridibunda) were used to indicate different haplotypes. The pink shaded rows show the newly described haplotypes, while the green lines represent the boundaries of SHGs.

Table 3-1. MHGs, haplotypes, source populations of haplotypes.

Haplogroup	H.ID	Sample ID	P.ID
ANATOLIACA MHG	CER1-10	CA07217, Hotz17207	17
	CER1-11	AKCA48213, AKCA48216	16
	CER1-12	HS07165, HS07166, AKCA48214, CA07218, CA07219	20, 16, 17
	CER1-21	AKCA48215	16
	A1-10	TBOECA68170, TBOECA68171, TBOECA68172, TBOECA68173, KKOBCA06230, KKOBCA06231,AJ310337	5, 6
	A1-11	CBCAST421, CBCAST3215, CBCAST3233, OECA68174, CBCA03184, CBCA03186, SGCA42199,	8, 29, 10, 5, 7
	A1-21	MACA22183	30
	A1-22	CBCA03188	7
	A1-23	ATDC08191, ATDC08192, ATDC08193, ATDC08194	31
	A2-10	CBCAST2021, CBCAST2022, CBCAST2023, CBCAST2024, CBCAST2026, CBCAST2027, CBCAST2028, CBCAST2029, CBCAST2031, CBCA6444, CBCA6445, CBCA6446, ISCA1578, ISCA1582, ISCA1583, ISCA1584, ISCA1585, ISCA1586, ISCA1587, ISCA1588, VDBKCA07126, MHSACA15137, YECA15146, KYECA15147, KYECA15148, MAHACA15152, CCYZCA20153, CCYZCA20155, CCYZCA20156, CCYZCA20157, CBCA03185, CBCA03189	14, 12, 26, 23, 25, 24, 15, 7
	A2-11	MAHACA15151	24
	A3-10	AKCA48209, AJ313131	18
	A3-11	AKCA48210, AKCA48211	18
	A3-12	AKCA48212	18
CEYHANENSIS MHG	CEY1-10	TBCASE0154, TBCASE0155, TBCASE0159, TBCASE0166, AJ313135	1
	CEY1-11	TBCASE0164	1
	CEY1-21	TBCASE0156, TBCASE0161, TBCASE0162, SOCA33224, SOCA33225, SOCA33226, SOCA33227	1, 2
	CEY1-31	TBCASE0157, TBCASE0158	1
	CEY1-32	TBCASE0160	1

Haplogroup	H.ID	Sample ID	P.ID
	C1-10	CBCAST072, CBCAST074, CBCAST076, CBCAST327, CBCAST328, CBCAST329, CBCAST3210, CBCAST3211, CBCAST3212, CBCAST3213, BCAST3214, CBCAST1517, CBCAST1519, CBCAST1520, CBCAST2025, CBCAST3234, CBCA3241, CBCA0743, MTAECA1568, MTAECA1569, MTAECA1570, MTAECA1571, MTAECA1573, MTAECA1576, MTAECA1577, ISCA1580, ISCA1581, OAOBBKCA1593, OAOBBKCA1594, OAOBBKCA1596, OBBKCA1597, OBBKCA1599, OBBKCA15101, OBBKCA15102, OBBKCA32104, OBBKCA32105, OBBKCA32106, OBBKCA32107, OBBKCA32108, HKHSBKCA07112, HKHSBKCA07113, HKHSBKCA07114, HKHSBKCA07115, HKHSBKCA07116, HKHSBKCA07119, HKHSBKCA07122, HKHSBKCA07124, VDBKCA07125, VDBKCA07127, VDBKCA07131, VD07134, VD07135, VD07136, MHSACA15138, MHSACA15140, MHSACA15141, MHSACA15142, MHSACA15143, MTHTCA07177, MTHTCA07178, OYCA70204, OYCA70206, AJ310314	22, 28, 11, 14, 10, 29, 27, 26, 23, 21, 25, 19, 4
	C1-11	CBCA3242	29
	C1-12	OBBKCA1598	28
	C1-13	HKHSBKCA07111, HKHSBKCA07123	22
	C1-14	HKHSBKCA07118	22
CARALITANA	C1-15	HKHSBKCA07121	22
MHG	C1-16	VDBKCA07128, MHSACA15139	23, 25
	C1-17	OYCA70206	4
	C1-18	OYCA70208	4
	C1-21	HKHSBKCA07120	22
	C3-10	CBCAST3216, CBCAST3238, CBCAST3239, CBCA2048, CBCA2051, MTAECA1572, MTAECA1575, AJ313132	29, 10, 13, 27, 9
	C3-11	CBCAST075	22
	C3-12	KYECA15150	24
	C4-10	CBCAST4240, CBCA2047, CBCA2050, CBCA2052, MTAECA1574, ISCA1579, VDBKCA07129, VDBKCA07130, YECA15145, KYECA15149, OECA68175, OECA68176, CBCA03187, CBCA03190, SGCA42202, AJ313133	7, 13, 27, 26, 23, 24, 5, 7, 8, 9
	C4-11	CBCA2049	13
	C2-10	AJ310316	9
	C2-11	MEFUCA42196, MEFUCA42197, MEFUCA42198, SGCA42201, OKCA42203, OK42220, OK42221, OK42222	9, 8, 3
	C2-21	MEFUCA42195	9
	C5-10	YECA15144, HS07167, HS07168, HS07169	24, 20
RIDIBUNDA MHG	RD1-10	MACA22179, MACA22180, MACA22181, MACA22182	30

Among 39 haplotypes, 30 haplotypes were described for the first time in this study. 15 of them belong to Caralitana MHG, 11 of them belong to Anatoliaca MHG, 4 of them belong to Ceyhanensis MHG, and only 1 was found in

Ridibunda MHG (Table 3.2 except Ridibunda MHG since it has only one haplotype).

Table 3-2. Number of individuals, number of haplotypes (unique types) and summary of standard and molecular diversity measures for each MHGs and whole data.

MHG	SHG	Number of individuals (proportion, %)	Number of haplotypes (unique types)	Number of segregating sites	Haplotype diversity	Mean number of pairwise differences	Gene diversity over loci
C	C1	101(87.8)	15 (12)	4.137 SD= 1.293	0.674 +/- 0.045	2.048 +/- 1.158	0.006 +/- 0.003
	C2	10 (8.7)	3 (2)				
	C5	4 (3.5)	1(1)				
A	A	57 (85.1)	10 (8)	3.550 SD= 1.236	0.755 +/- 0.050	2.918 +/- 1.550	0.008 +/- 0.005
	Cer	10 (14.9)	4 (3)				
CEY		16	5 (4)	1.507 SD= 0.826	0.733 +/- 0.079	1.467 +/- 0.937	0.004 +/- 0.003
RD		4	1	-	-	-	-
WHOLE DATA		**203**	**39 (30)**	**9.341 SD=2.336**	**0.867 +/- 0.017**	**7.784 +/- 3.640**	**0.022 +/- 0.012**

1. Caralitana MHG: Assuming it is a reliable marker of the form *caralitana*, the form's range was extended from İvriz - Konya to western borders of Burdur province, Işıklı Lake - Denizli and from Esmekaya - Aksaray in the northeast to Avlan Lake, including the whole coastal region of Antalya east to Silifke (İçel) (Figure 3-3). Within MHG, haplotype diversity is about 0.674; however, the mean number of pairwise differences is smaller (2.048) when compared to whole data set because of the presence of many haplotypes having very closely similar sequences. As a result of pairwise comparisons of the mt ND3 sequences of Caralitana with Anatoliaca, Ceyhanensis, the number of substitutions (transitions + transversions) ranges between 6 - 16, 13 - 19, corresponding to pairwise distances (d_{JC} distance) 0.018 - 0.049, 0.039 - 0.058, respectively (appendix). Within Caralitana MHG nucleotide substitutions change from 1 to 9

(0.003 - 0.027 of parwise distance). A greater distance than 0.018 (more than 6 substitutions) is due to C2 and C5 SHGs and the reason for a split into 3 sub-haplogroups.

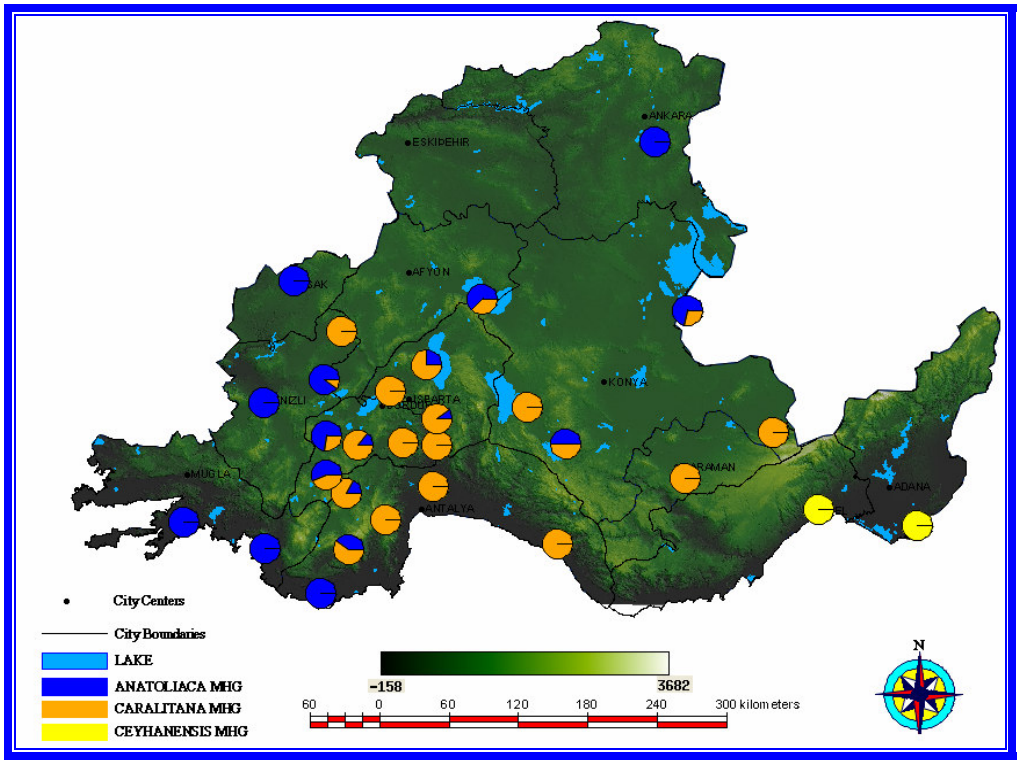


Figure 3-3. Map showing distribution and proportions of mtDNA mainhaplogroups in the study site. Orange: Caralitana; Blue: Anatoliaca; Yellow: Ceyhanensis (Kaya, 2006).

1.1. Caralitana1 SHG (C1 SHG): It is the most widespread and older group within Caralitana MHG, predominantly found within the Antalya province, especially eastern parts as well as in Karaman and Isparta (Figure 3-4). Its range extends to western borders of Burdur province, and includes eastern parts of Denizli and southern parts of Tuz Lake. When comparing C1 SHG with C2,

and C5 SHGs, substitution values 4 - 9 (d_{JC} = 0.012 - 0.027), 4 - 8 (d_{JC} = 0.012 - 0.024) are clearly seen. Within SHG, pairwise distances range within 0.003 - 0.015, corresponding to substitution values of 1 - 5.

1.2. Caralitana2 SHG (C2 SHG): This SHG was only found in the Konya plain and Lake Beyşehir, the type locality of *caralitana* taxon (Figure 3-4). Distance and number of substitutions between C2 SHG and C5 SHG, C1 SHG represent the highest values within MHG: 8 - 9 (d_{JC} = 0.024 - 0.027), 4 - 9 (d_{JC} = 0.012 - 0.027). The populations are geographically localized and isolated from other Lake District populations; however, they coexist syntopically with haplotypes belonging to C1 SHG. Thus, they might exhibit a pairwise distance between intra- and inter-mainhaplogroup levels. Nevertheless, a more detailed investigation is required to understand the whole picture of this group.

1.3. Caralitana5 SHG (C5 SHG): This subhaplogroup was only recorded in southwestern part of Burdur and Avlan Lake - Antalya (Figure 3-4), the transition region between Caralitana MHG and the Cerigensis sub-haplogroup. It was found syntopically with Cerigensis SHG, C1 SHG and A1 SHG. The genetic distances and number of substitutions between C5 SHG and others (given above) ranged between 4 - 9 (d_{JC} = 0.012 - 0.027). The distance is again between intra- and inter-mainhaplogroup levels. For the definite geographic distribution of C5 SHG, further study is needed.

2. ANATOLIACA MHG: This main haplogroup was found in the surrounding regions of Caralitana MHG (Figure 3-3). Interestingly, one haplotype from Anatoliaca MHG was found in Edirne (European Turkey) where Ridibunda MHG exists while one other haplotype was recorded in Artvin population, in northeastern Turkey. These findings suggest a wide range Anatolica MHG. Within MHG, haplotype diversity is about 0.755, and the mean number of pairwise differences is 2.918, which are greater values than the same measures for Caralitana MHG. The number of nucleotide substitutions between Anatoliaca MHG and Caralitana MHG, Ceyhanensis MHG, are ranging between

6 - 16, 11 - 16, correspond to distance values 0.018 - 0.049, 0.033 - 0.049, respectively. Within Anatoliaca MHG, the genetic distance changes from 0.003 to 0.030 (1 - 10 substitutions). This greater value (>0.018) is due to the Cerigensis SHG. This MHG is further divided into 2 sub-haplogroups:

2.1. Anatoliaca1 SHG (A1 SHG): It is the most widespread and older group, identified mainly in Ankara, Akşehir - Eber, Eşmekaya - Aksaray, Artvin, Sorgun Dam - Uşak, Gökpınar Dam - Denizli, Muğla, and Edirne populations (Figure 3-4). A1 SHG was found largely parapatric to Caralitana MHG, showing a wide clinal variation on the southwestern part of Burdur. Nevertheless, their syntopic occurence was also recorded in Seydişehir - Konya , Eğirdir Lake - Isparta, Eşmekaya - Aksaray, and some populations in Burdur.
In addition, A2 coded haplotypes were predominantly recorded in western Anatolia, whereas A3 coded haplotypes were only found in Muğla. The genetic distances (d_{JC}) and number of substitutions between A1 SHG and Cerigensis SHG range between 0.012 - 0.030 (4 - 10). Within A1 SHG, pairwise distances change from 0.003 to 0.015 (1 - 5 substitutions).

2.2. Cerigensis SHG (Cer SHG): *Rana cerigensis* was described as a separate species by Beerli et al. (1994) that occurs in Karpathos and Rhodes Islands (Greece). Previously, there was only one record of a Cerigensis haplotype from Antalya (Pötner, 2005); however, this study, also found the haplotype in western coastal part of Antalya: Kaş, Avlan Lake populations and in Fethiye - Muğla (Figure 3-4). It seems that populations could be locally restricted in this region and allopatrically separated from A1 SHG and Caralitana MHG by mountain barriers. However, Cerigensis and C5 haplotypes were identified in the Avlan population, indicating that populations probably come into contact in the transition regions, but more comprehensive investigation is needed to reveal the degree of gene exchange with other groups, as well as the exact structure of the population. The pairwise distance between Cer SHG and A1 SHG has both lower and greater values than 0.018, the extent of these values were accepted as between intra- and inter-mainhaplogroup level.

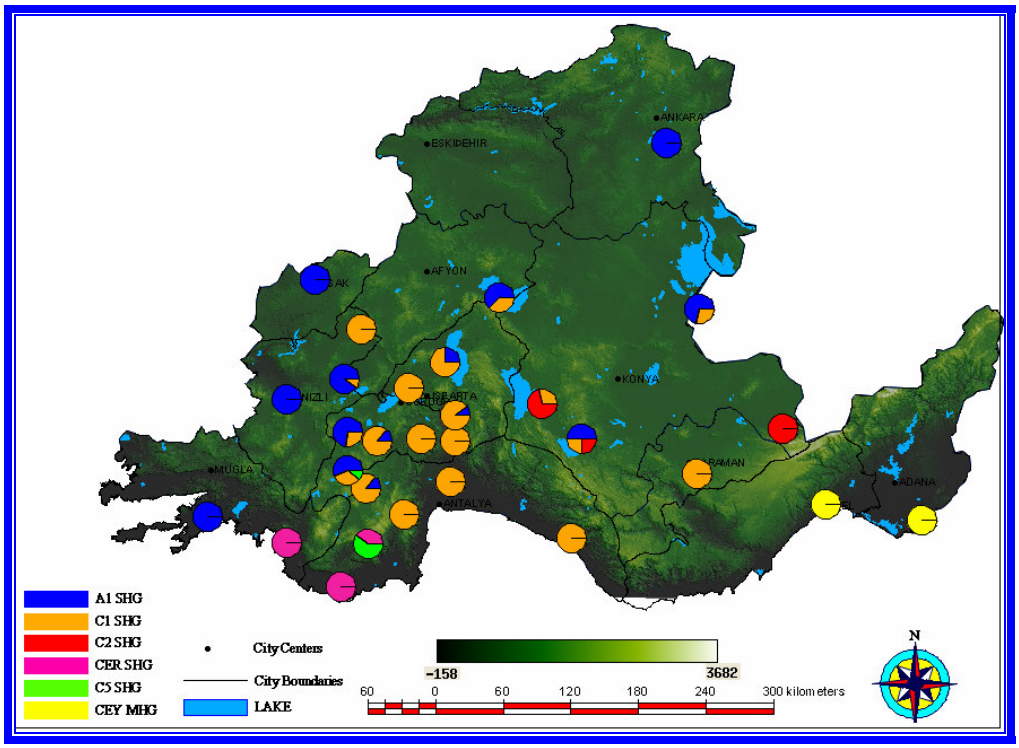


Figure 3-4. Geographic distribution and proportions of subhaplogroups within ranges of MHGs. Orange: C1; red: C2; green: C5; pink: Cerigensis; blue: A1; yellow: Ceyhanensis (Kaya, 2006).

3. CEYHANENSIS MHG: It was first recorded from Ceyhan (Adana) by Plötner et al. (2001). Together with this study, its range was extended to Mersin (eastern İçel province). Nevertheless, its northern, eastern and southern boundaries remain unclear. Pairwise distances between Ceyhanensis and Caralitana, Anatoliaca MHGs, are considerably larger comparing with values mentioned above: d_{JC} = 0.039 - 0.058 (13 - 19), d_{JC} = 0.033 - 0.049 (11 - 16).

4.RIDIBUNDA MHG: This main haplogroups was identified in the Edirne population. Pairwise distances between Ridibunda and Caralitana, Anatoliaca, Ceyhanensis MHGs range d_{JC} = 0.071 - 0.087 (23 - 28), d_{JK} = 0.058 - 0.071 (19 -

23), d_{JC} = 0.039 - 0.058 (13 - 19), d_{JC} = 0.055 - 0.064 (18 - 21), indicating that the Ridibunda taxon had separated from other Anatolian MHGs much long ago .

3.2.2. Phylogenetic Analyses Results

3.2.2.1. NJ Tree of Mt DNA Sequences of Turkish Water Frog Complex

An NJ tree was constructed based on Jukes and Cantor substitution model among haplotypes, which generally showed low bootstrap values. This probably arises from missing haplotypes in other parts of Anatolia or due to the high variability of genetic distances measured among haplotypes within MHGs. For the construction of phylogenetic tree, 4 of ND3 sequences from gene bank with accession numbers AJ310334 (Troodos, Cyprus), AJ310336 (*R. cretensis*, Crete), *R. epeirotica* Greece and Italian water frog (from J. Plötner's database) were combined to use as outgroups, and 4 ND3 sequences from Syria with accession numbers AJ310313, AJ310312 (Abu Kemal), AJ310322 (Qal'at al Hisn), AJ310320 (Burj Safita, Ansari mountains) were incorported to reveal the taxonomic relationship between Anatolian water frogs and *R. bedriagae.*

The obtained results from NJ tree indicate that Anatolian water frogs are divided into 3 clades (Figure 3-5): The first clade consists of Caralitana MHG, including populations from southwestern Turkey and Konya, where *R. ridibunda caralitana* was described as a separate subspecies by Arıkan (1988) and later suggested as a full species (Jdeidi et al., 2001; Plötner and Ohst, 2001).

The clade comprises Anatoliaca MHG, including Cerigensis SHG, samples from Abu Kemal (Syria) and the populations surrounding the Caralitana MHG in the rest of Asiatic Turkey. In previous studies, these populations were accepted as *R. bedriagae* Camerano 1882, whose type locality is Damascus (Syria). However, Anatoliaca MHG is clearly different from real *R. bedriagae* clade, whose samples

had come from its type locality. Remarkably, samples from Syria - Abu Kemal are far away from the type locality of *R. bedriagae*, probably not more divergent than SHG Cerigensis within the Anatoliaca MHG Therefore, all of these results support that Anatoliaca MHG does not belong *R. bedriagae* species as suggested by Plötner and Ohst (2001).

The third clade is formed by Ceyhanensis MHG which includes Adana and (eastern) İçel. This clade branched off earlier than the other Anatolian clades (Anatoliaca and Caralitana MHGs) and represents a phylogenetically older lineage and undefined taxon. These results are also supported by Plötner and Ohst (2001).

Rana ridibunda clade, found in European part of Turkey, is clearly divergent from the Anatolian water frog clades, indicating an older clade among Turkish water frog groups.

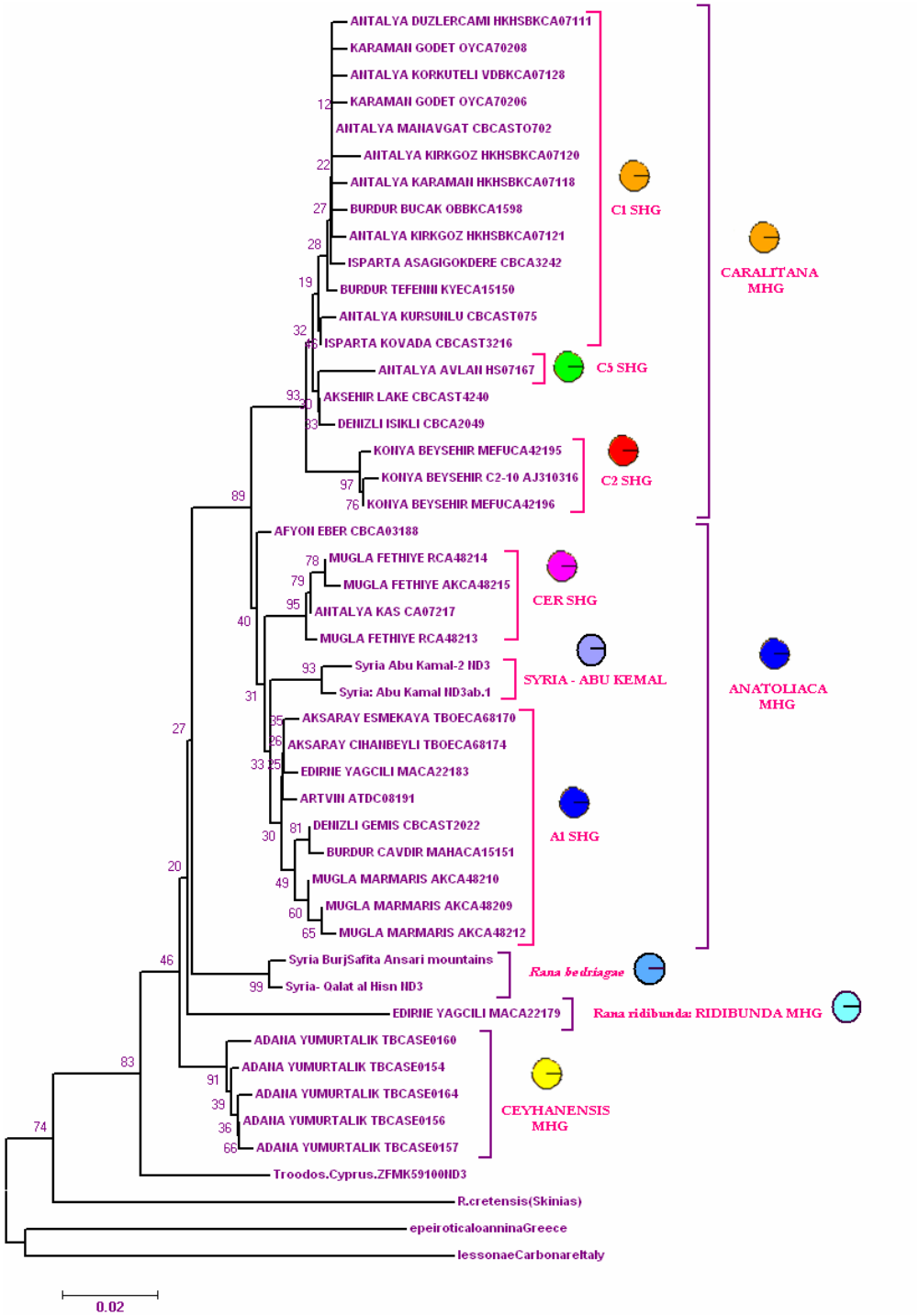


Figure 3-5. Genetic relationships of mt DNA MHGs of Turkish Water frogs calculated on the basis of Jukes and Cantor distances using Neighbour-Joining algorithm. Numbers on branches indicate bootstrap values.

3.2.2.2. Minimum Spanning Network

To reveal relationships among closely related sequences of haplotypes, MSN was constructed using Arlequin software 3.01 based on pairwise differences among them.

Firstly, MSN was drawn for Caralitana MHG shown in Figure 3-6. Caralitana MHG had 19 different haplotypes, collected from a totaly 21 distinct populations. 15 of them were newly described in this study. C2 SHG and C5 SHG are geographically localized, found in 3 and 2 different populations, respectively, while C1 SHG is the most variable and common haplotype and found in 17 populations. The relationships of 3 SHGs are clearly seen; they are connected to each other through at least 4 mutational steps and this also supports the definition of 3 such sub-groups.

C1-10 is the most widespread haplotype, recorded from 13 different populations with a high frequency (n=63). It is an interior haplotype connected to 10 rare haplotypes, having many mutational connections. Additonally, C4-10 and C3-10 are other common haplotypes, collected from 9 and 5 different populations, respectively. Therefore, we suggest that C1-10 is the oldest haplotype among them since it must have been present in the populations for a longer time to be range so widely and to have a considerable copy number (Watterson et al., 1977; Donnelly and Tavare, 1986; Excoffier and Smouse, 1994). In addition, haplotypes with a high copy number have a greater probability of becoming interior haplotypes and are more likely to have more mutational connections. The rare haplotypes (singletons) derived from common haplotype represent more recent mutations and they are more related to common haplotypes rather than rare ones (Excoffier and Langaney, 1989).

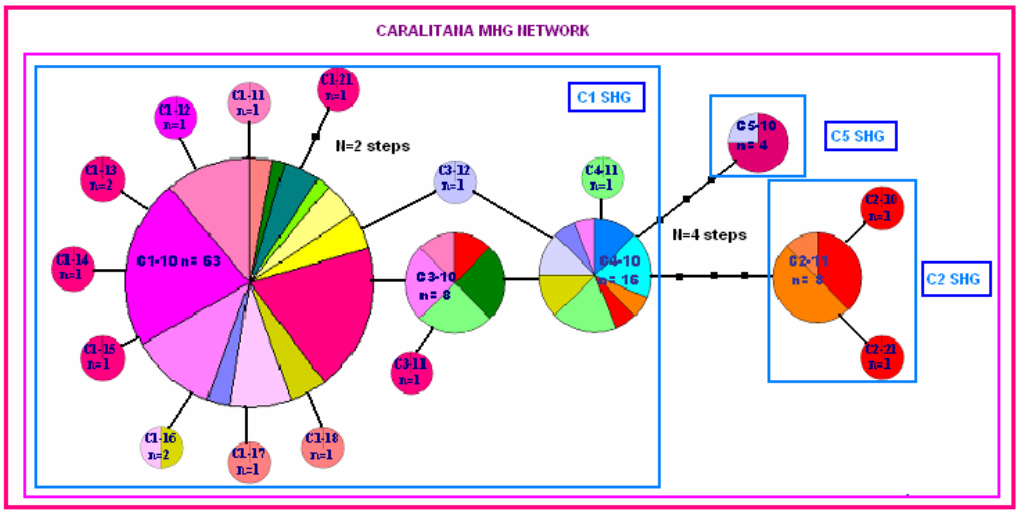


Figure 3-6. MSN of Caralitana mainhaplogroup. The circles represent haplotypes and the number of mutational steps are denoted by a small square on the lines connecting them. If there is only one mutation between two haplotypes, then they are connected with a single line. The size of circles are drawn proportional to the number of haplotypes obtained in the study. Different colors indicate a particular population.

Secondly, MSN was drawn for Anatoliaca and Ceyhanensis MHGs shown in Figure 3-7. Anatoliaca MHG has 14 different haplotypes, recorded from 19 distinct populations. 11 of them were newly described in this study. Cerigensis SHG was geographically restricted, obtained from 3 populations while A1 SHG is highly variable and widespread, recorded from 16 different populations. In A1 SHG, A2-10, A1-11, A1-10 are the most common haplotypes, obtained from 8, 5, 2 different populations, respectively. Cerigensis and A1 SHGs are connected to each other by 4 mutational steps, supporting the previous results again.

Ceyhanensis MHG has 5 distinct haplotypes, 4 of them were described in the present study, collected from only 2 populations.

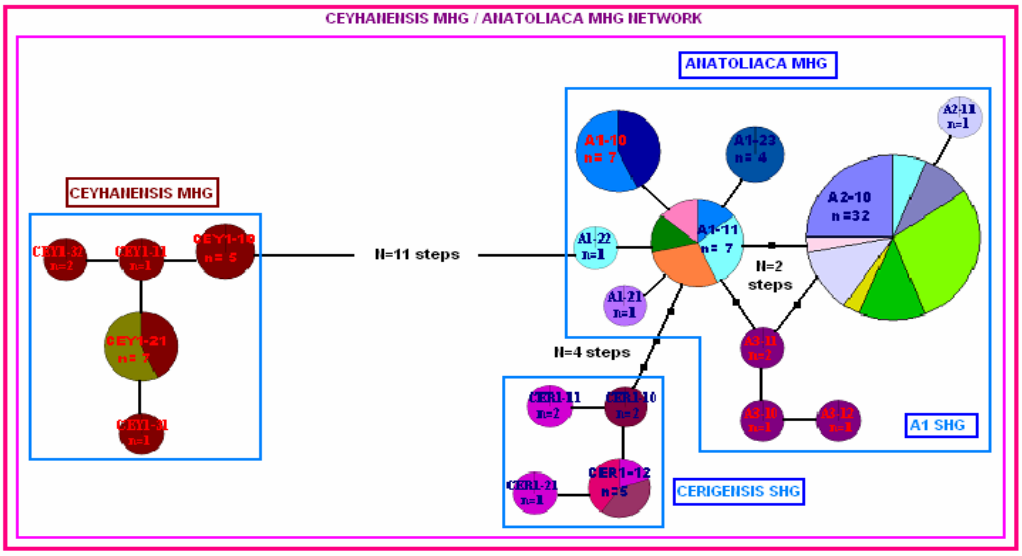


Figure 3-7. MSN of Anatoliaca and Ceyhanensis mainhaplogroups. See Figure 3-6 for explanation.

As a next step, MSN was drawn for Caralitana MHG and surrounding MHGs (Anatoliaca and Ceyhanensis) to reveal the close relationships among them and shown in Figure 3-8. The distinction among 3 MHGs is clearly seen. Caralitana and Anatoliaca MHGs are connected by 6 mutational steps, corresponding to d_{JC}=0.018 genetic distance value (the smallest value between them), and Anatoliaca and Ceyhanensis MHGs are connected by 11 mutational steps, corresponding 0.033. Within MHGs, both A1 SHG and Cerigensis SHG , and C1 SHG and C2, C5 SHG are connected to each other by 4 mutational steps, corresponding d_{JC}= 0.012 genetic distance. This means that Ceyhanensis is probably the older group and diverged earlier than 2 other MHGs whereas Caralitana and Anatoliaca are more closely related to each other. Consequently, MSN results confirm both the presence of 3 distinct Anatolian MHGs (Caralitana, Anatoliaca and Ceyhanensis) and the separation of SHGs within Caralitana MHG (C1, C2 and C5 SHGs) and Anatoliaca MHG (A1 and Cerigensis SHGs).

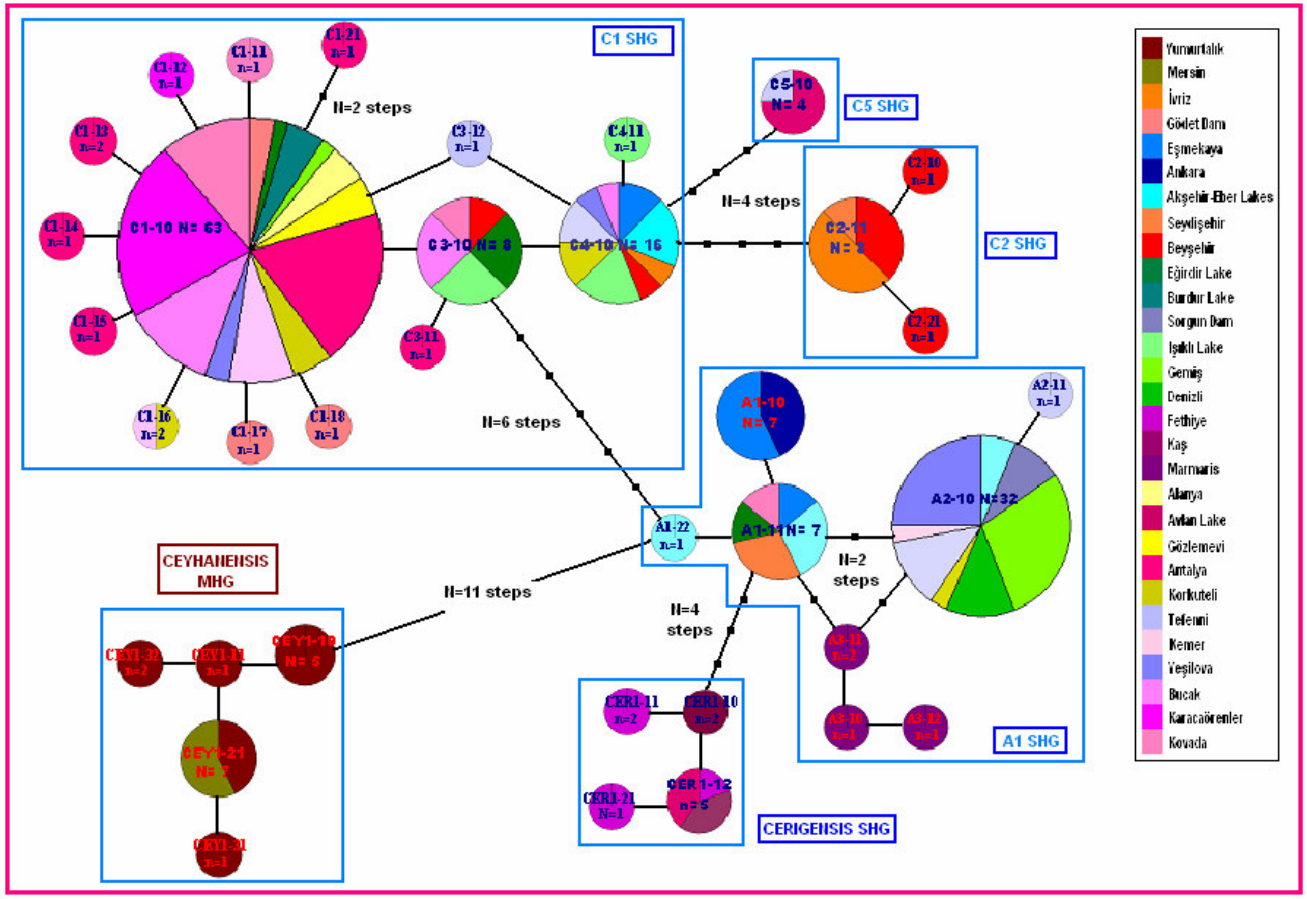


Figure 3-8. MSN of Caralitana MHG and its surrounding MHGs (Anatoliaca and Ceyhanensis). See Figure 3-6 for explanation.

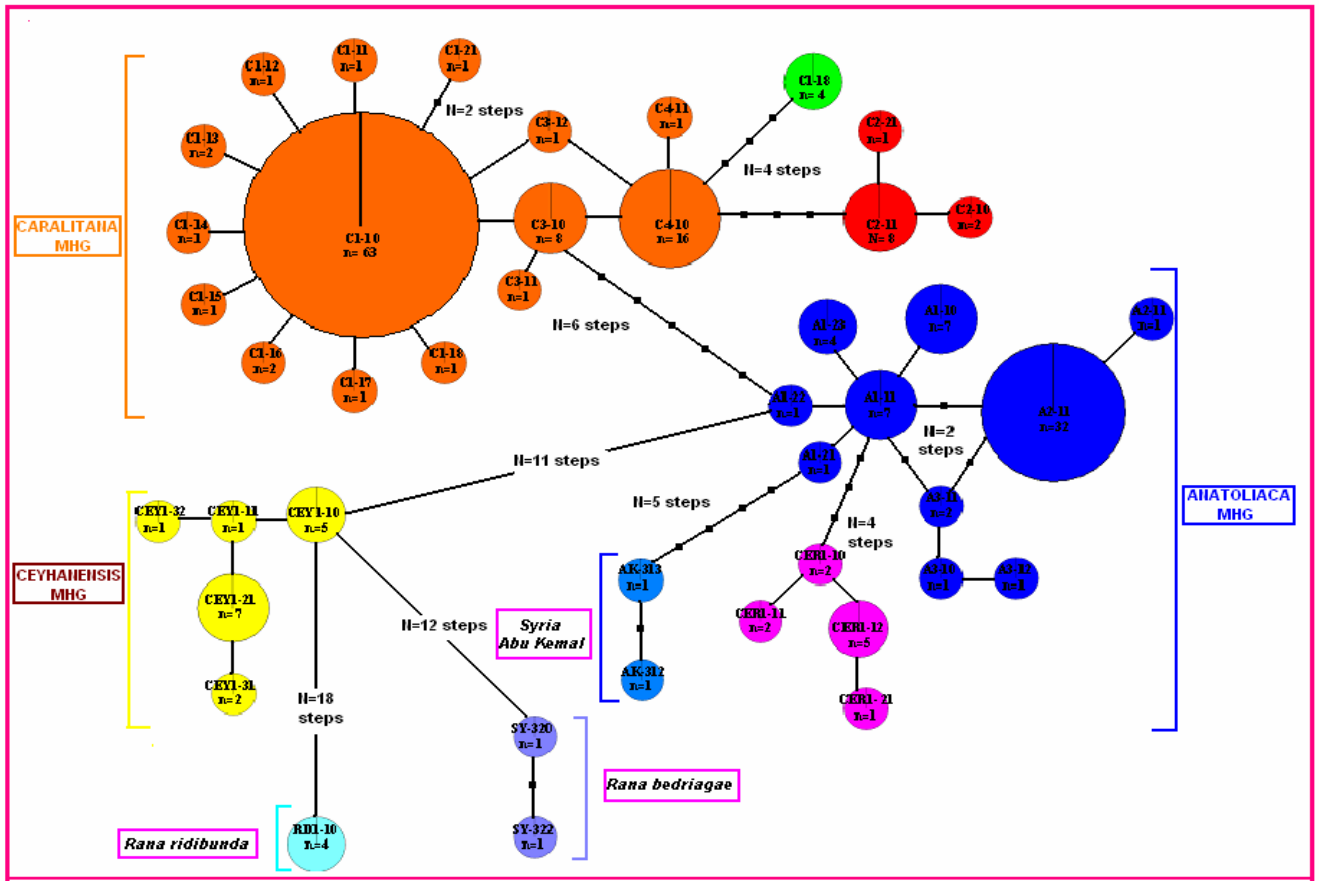


Figure 3-9. MSN of haplotypes obtained from Turkish water frogs together with 4 Syrian samples. See Figure 3-6 for explanation. SYrepresents *R. bedriagae* from Damascus (SW Syria) while AK represents Abu Kemal (NE Syria).

Finally, MSN was drawn for all Turkish water frogs haplotypes including 4 samples for *R. ridibunda* from Thrace and 4 samples from Syria, used as outgroup samples. Apparently, *R. ridibunda* is connected to Anatolian water frogs through 18 mutational steps while *R. bedriagae* is connected to Anatolian water frogs by 12 mutational steps. This strongly supports that Anatoliaca MHG does not belong to *R. bedriagae* (Plötner and Ohst, 2001). However, as previously noted, the 2 samples from Abu Kemal (NE Syria) are connected to the Anatoliaca MHG by at least 5 mutational steps, i.e. closer to Anatolian water frogs than *R. bedriagae*. Abu Kemal is quite far from the type locality of *R. bedriagae* in southwestern Syria. It is probably connected to Eastern Anatolia by the Euphrates river.

3.2.3. The Genetic Structure of Anatolian Water Frog Populations: SAMOVA

To identify the genetic structure of groups of populations and objectively test whether their validity, SAMOVA 1.0 was used (Dupanloup et al., 2001). The program defines geographically homogeneous and maximally differentiated groups of populations by maximizing the proportion of total genetic variance due to differences between groups of population (i.e. F_{CT} is maximized).

SAMOVA was run using 100 simulated annealing processes for K=2 to K=9. The best grouping scheme was obtained for K=5 with F_{CT}= 0,68223 (***P<0,001) where a rapid increase are seen in F_{CT} values up to K=5 (Figure 3-10). Although, there is still an increase F_{CT} for K> 5, the value is not significantly different from the previous, due to weak differences among groups.

SAMOVA results clearly indicate the structure of groups summmarized in Table 3-3. Group 1 consists of 2 distinct populations, exclusively represented by Ceyhanensis haplotypes (100 %). Similary, group 4 with 2 different populations contains only pure Cerigensis haplotypes (100 %).

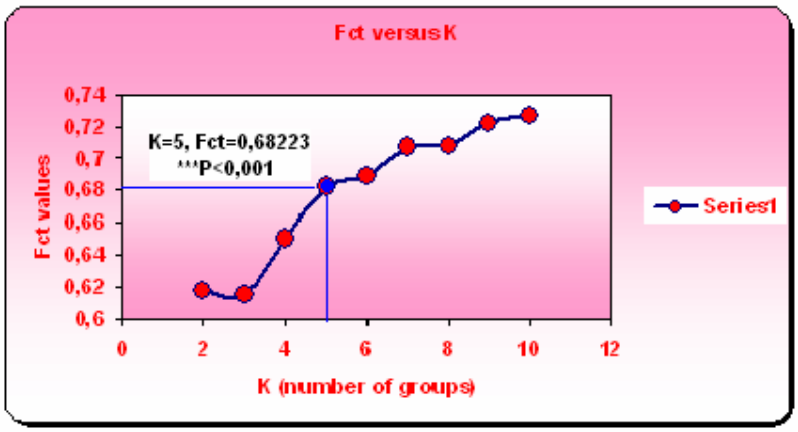


Figure 3-10. *K* (number of groups) versus F_{CT} values obtained from SAMOVA analysis.

Group 2 includes 10 different populations, mostly represented by haplotypes belonging to A1 SHG (76,60 %). Interestingly, the rest of haplotypes (23,40 %) belonged to Caralitana MHG and were found in the localities where sharp genetic changes occur and at least in one of which introgression is clearly seen.

Group 3 with 12 distict populations is nearly represented by haplotypes belonging to C1 SHG (90.53 %) and only few haplotypes that belong to C5 SHG (3.16 %). In the rest of haplotypes, 4.21 % of haplotypes belonging to A1 SHG were found only in populations where introgression is seen, and 2.11% of haplotypes belonging to Cerigensis SHG were found in a population where sharp genetic change occurs.

Finally, group 5 consists of 2 distinct populations, mainly represented by C2 SHG (81,80 %). The rest of haplotypes belong to C1 SHG (18,20 %).

Table 3-3. Results of genetically differentiated groups of populations obtained from the SAMOVA algorithm.

Group Number	Populations	corresponding haplogroup	% value for haplogroup	Fct value
Group 1	Yumurtalık Mersin	Ceyhanensis MHG	100%	0,68223 ***P<0,001
Group 2	Eşmekaya Ankara Akşehir-Eber L. Seydişehir Sorgun Dam Gemiş Denizli Marmaris Tefenni Yeşilova	Anatoliaca1 SHG Caralitana1 SHG Caralitana2 SHG Caralitana5 SHG Cerigensis SHG	76, 60% 20,31% 1,56% 1,56% 0,0%	
Group 3	Gödet Dam Eğirdir Burdurlake Işıklılake Alanya Avlanlake Antalya Korkuteli Kemer Bucak Karacaören Kovada	Anatoliaca1 SHG Caralitana1 SHG Caralitana2 SHG Caralitana5 SHG Cerigensis SHG	4,21% 90,53% 0,0% 3,16% 2,11%	
Group 4	Fethiye Kaş	Cerigensis SHG	100%	
Group 5	Beyşehir İvriz	Caralitana1 SHG Caralitana2 SHG	18,20% 81,80%	

Consequently, results obtained from SAMOVA strongly support the previously defined genetic structure of all main haplogroups and their sub haplogroups, except for Caralitana5 SHG. This is probably due to small sample size in the geographic area where C5 haplotypes were recorded. In addition, all groups show a significant geographic structure (Figure 3-11). Group 1 is separated from group 3 by the Middle Taurus Mountains. Group 4 is separated from group 2 and group 3 by Gölgeli, Boncuk and Bey Mountains. Group 3 restricted to central south Turkey is separated from group 2 on the north (from west to east) by Sultan Mountains, the Bozdağ Massif, and Karaca and Melendiz mountains. However, on the western borders of territory of group 3 there are no obvious geographic barriers and a hybrid zone is more apparent than elsewhere.

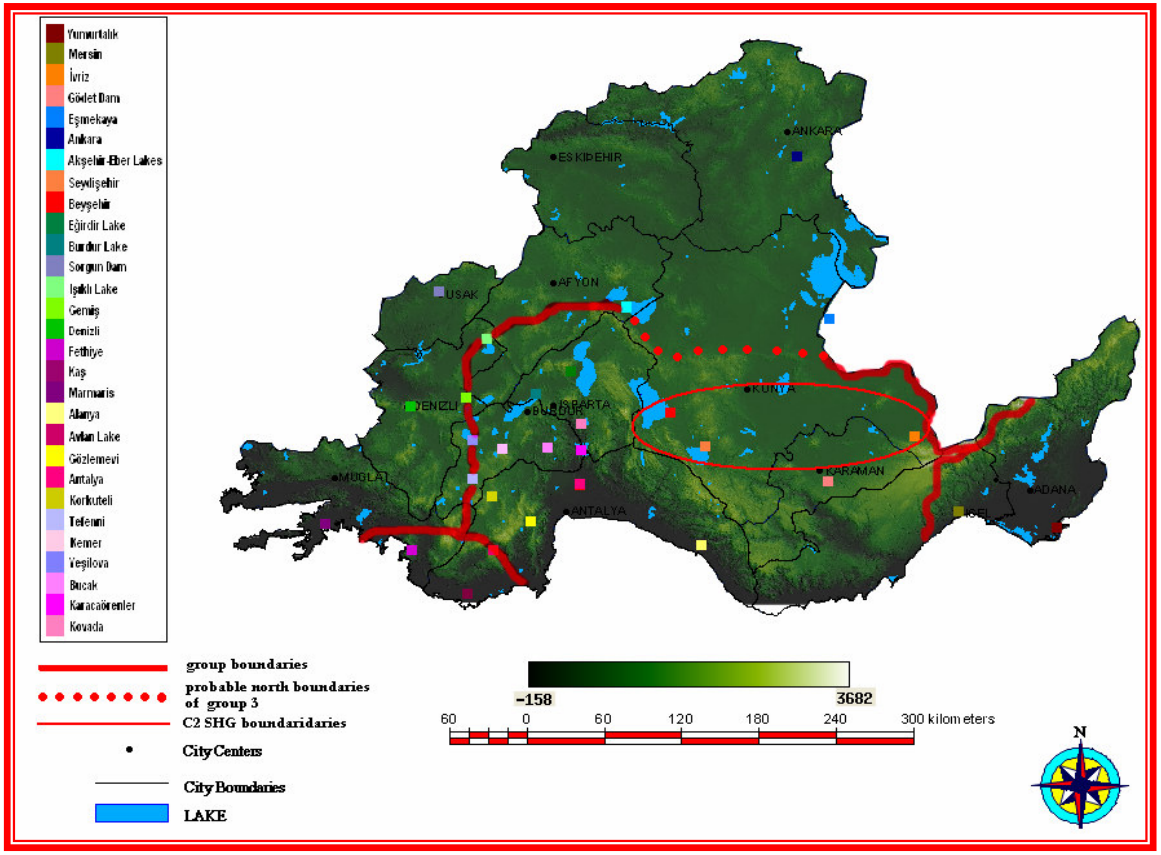


Figure 3-11. The genetic structures and boundaries (sharp genetic changes occur) of groups obtained using SAMOVA algorithms (Kaya, 2006).

Within the Konya plain group 5 and group 3 (both belong to the Caralitana MHG) are sympatric. These findings suggest that apart from physical geographic barriers such as high mountains, there might be other reasons such as pre- zygotic (e.g. assortative mating) or post-zygotic (e.g. lowered hybrid survival or reproduction) isolation mechanisms, association between alleles, or reduction in fitness due to dispersal/selection balance (Barton and Hewitt, 1985) that lead to observed genetic change among largely parapatric populations.

3.2.3. Geographic Subdivision In Anatolian Water Frog Populations: AMOVA

To see the haplotypic diversity at the different level of hierarchy, AMOVA was carried out using Arlequin 3.01 software, where molecular variance obtained from distance between haplotypes are partitioned among groups, populations and populations within groups. The genetic structure of 5 groups resulted from SAMOVA algorithm was directly subjected to AMOVA. In Table 3-4, results indicate that at all levels of the hierarchy, the differences are statistically highly significant (***$P<0.001$). Furthermore, the greatest part (68.22 %) of the total molecular variance was partitioned among groups, supporting again genetically and geographically well differentiated groups of populations due to the presence of arrays of haplotypes and effective geographic barriers (mountains) (Excoffier et al., 1992) and also the branching pattern of MSN.

Remarkably, the relatively high variation (24.47 %) observed within populations is probably caused by populations at or near taxon boundaries, containing haplotypes belonging to two or more MHGs or SHGs whereas the much lower variation (7.31 %) among populations within groups might reflect more homogenous populations consisting mostly of haplotypes different from one another by a few mutational steps. Therefore, the AMOVA results explain

clearly the population subdivision in southwestern Turkey and verify previous findings.

Table 3-4. AMOVA results of genetically differentiated groups of water frog populations.

Source of Variation	df	Variance Components	Percentage of Variation	Fixation Indices
Among groups	4	3.23813	68.22	Φ_{CT}=0.68223 ***
Among populations within groups	24	0.34674	7.31	Φ_{SC}=0.2299 ***
Within populations	165	1.16152	24.47	Φ_{ST}=0.75528 ***
Total	193	4.74639		

***P<0.001

3.2.4. Historical Demography of Water Frog Populations

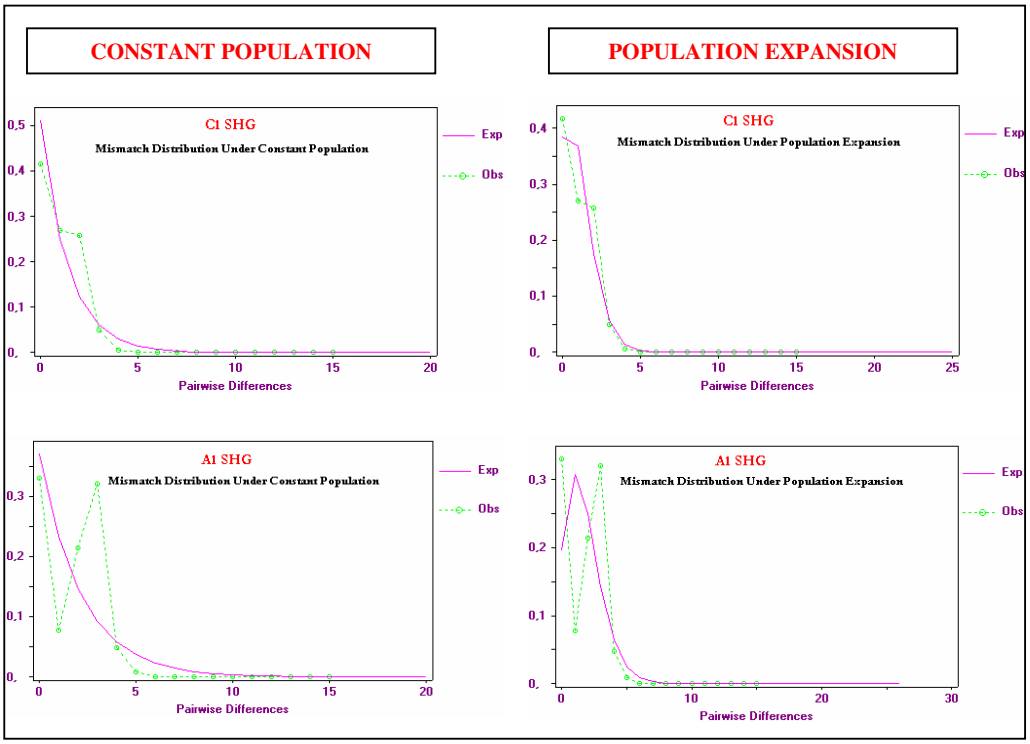
3.2.4.1. Mismatch Distribution

Mismatch distribution (the distribution of nucleotide substutitons pairwise differences) is used both as a measure of genetic diversity and an indicator of past population history. The analysis was conducted and drawn using DnaSP software only for C1 SHG and A1 SHG separately since the rest of groups have not adequate a sample size to make an inferences about their past demography.

In figure 3-12, the graphs reflect the observed and expected mismatch distributions, drawn both under the assumption of constant population size on

the left, and the assumption of population expansion on the right. The observed distribution fit expected in C1 SHG for the assumption of population expansion whereas the observed distribution of A1 SHG fit the distribution neither under the assumption of constant population nor the assumption of population expansion. Remarkably, the unimodal shape distribution of C1 SHG show the best fit under population expansion; thus, this SHG could be subjected to population expansion at the past. Especially, results obtained from MSN for C1 SHG clearly reveal a star like phylogeny having many short internal branch lenghts and an excess of singleton mutations on the terminal branches, which can be good indicators for population expansion (Ray et al., 2003; Rogers and Harpending, 1992; Harpending, 1994; Slatkin and Hudson, 1991; Schneider and Excoffier, 1999).

Figure 3-12. Observed and expected mismatch distributions of five water frog haplogroups under constant population (left) and population expansion (right).



The mean number of differences for A1 SHG is 1.7036, which is higher values comparing to C1 SHG with 0.9585. Therefore, A1 SHG is probably older originated and dispersed population since the number of haplotypes used in mismatch construction for this group are smaller than C1 SHG, but reflecting a greater genetic diversity.

3.2.4.2. Neutrality Tests

To make inferences the historical demography of C1 and A1 SHGs, more powerful tests were conducted using DnaSP software.

The neutrality test results are shown in Table 3-5. The gene diversities are lower compared to haplotype diversities, which probably reflects recent population expansion. Furthermore, only for C1 SHG, significant negative results were obtained from both Li and Fu's F* and D* and Fu's Fs tests. According to Fu (1997) and Ramos-Onsins et al. (2003), Fs is the most powerful test to detect the population growth and genetic hitchhiking based upon significant negative results and the presence of large sample size. However, genetic hitchhiking leads to the fixation of a single haplotype in the population and shows significant negative values for Tajima's D test (Charlesworth et al., 1993), which were not observed in C1 SHG. Therefore, C1 SHG has probably experienced population growth, which was also supported the unimodal shape of mismatch distribution and star like phylogeny with so many singleton mutations on the external branches. Thus, population expansion is the best inference to be suggested for C1 SHG (Ray et al., 2003; Rogers and Harpending, 1992; Harpending, 1994; Slatkin and Hudson, 1991; Schneider and Excoffier, 1999).

Lastly, for A1 SHG significant results could not be obtained either because it was not subjected to population expansion and there may be other selective

constrains, or sample size is not adequate to make an inference about past demographic history of populations. Hence, more comprehensive studies or other population models are clearly needed.

Table 3-5. Diversity estimates and neutrality test results obtained from two water frog haplogroups for mitochondrial ND3 gene region.

	C1 SHG	A1 SHG
Number of haplotypes	15	10
M. number of pairwise differences	0,9584	1,7036
Gene diversity over loci	0,0028	0,0050
Haplotype diversity	0,5836	0,6697
Raggadness (r)	0,0665	0,1689
Tajima's D test (1989)	-1,0276	-0,5889
Fu and Li's F* test (1993)	**-3,9012 ***	-1,1312
Fu and Li's D* test (1993)	**-4,1330 ***	-1,1429
Fu's Fs (1997)	**-11,078 ****	-2,3569

*P<0,05 ** P<0,01

3.3. Concordance Between Ventral Color and MtDNA Haplotypes

Orange colored maculation on the venter has been used to identify the *caralitana* form in many previous studies (Atatür et al., 1889 -1990; Arıkan et al., 1994; Arıkan et al., 1998; Budak et al., 2000; Jdeidi et al., 2001; Kaya et al., 2002; Düşen et al., 2004; Tosunoğlu et al., 2005). Therefore, to evaluate the taxonomic value of orange venter and understand whether there is any correlation between orange colored maculation and Caralitana haplotypes, the geographic distributions of both parameters were combined in each river basin and presented in Figure 3-13.

Results show that in the river basins 5, 6, 8, 12, within the geographic range of C1 SHG, these two parameters do not coincide well; that is, several individuals with Caralitana specific haplotypes had white colored venters. Furthermore, in the remaining river basins, generally ventral color and mtDNA haplotypes were concordant with each other, except for some individuals in the transition regions or in regions where rapid genetic change occurs. It means that several individuals with Anatoliaca or Cerigensis specific haplotypes have orange colored maculation or individuals with black-brown or white maculation carry Caralitana specific haplotypes. This probably occurs due to introgression among different water frog maternal lineages.

Consequently, our results support that orange colored maculation generally is not concordant with C1 SHG within the geographic range of that lineage. Therefore, it is not reliable by itself to diagnose any *caralitana* form alone on the basis of ventral color and combination of other characters is required for taxonomic characterization and identification.

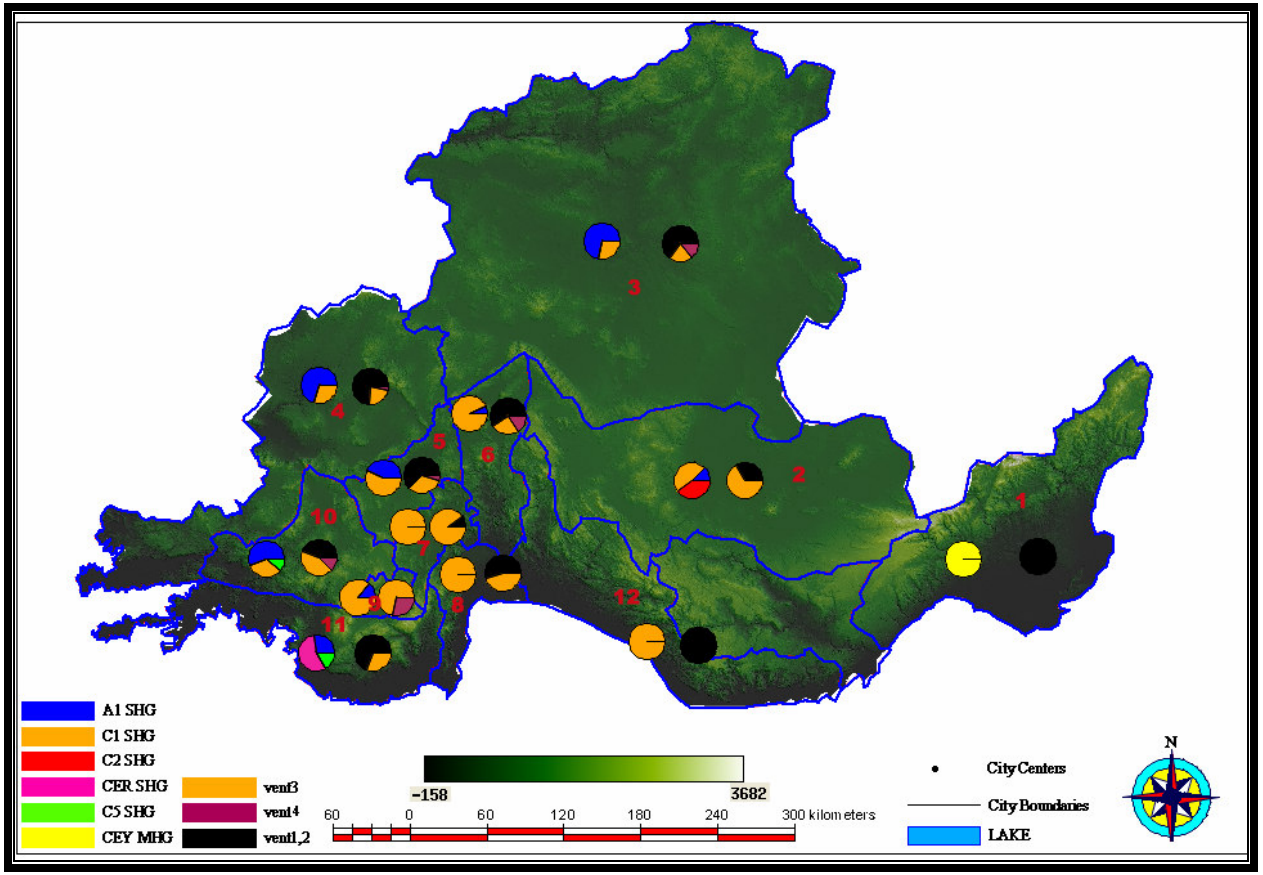


Figure 3-13. Concordance between ventral color and mtDNA haplotypes in the river basins (Kaya, 2006).

3.4. Introgression and Hybrid Zone

MtDNA results suggest that a narrow hybrid zone exists where A1 SHG and C1 SHG meet at the northern and eastern borders of the Lake District (Figure 3-14). There introgression is evidently asymmetrical from A1 SHG populations to C1 SGH populations. A female-biased dispersal from A1 SHG into C1 SHG populations is probable since male frogs of the Caralitana group may have mating advantage with Anatoliaca-type females over other males due to their big size (Jdeidi et al 1999). Furthermore, because of lack of parental care in frogs, in order to maximize reproductive success, females avoid inbreeding, which makes female-dispersal advantageous. Hence, they should prefer to mate males from other populations (Austin et al., 2003).

However, in the Konya plain, introgression is seen in both directions, both from Anatoliaca to Caralitana group and vice versa. The observed reciprocal introgression may be the outcome of a long slow process of dispersal since there are few physical obstacles that would act as isolating barriers in southern Central Anatolia. Alternatively, or perhaps additionally, a contemporary human-made canal may have acted as a dispersal corridor. A nearly 150 km long irrigation canal was constructed in 1974 that connected Beyşehir Lake to Tuz Lake through Suğla - Çumra. The canal might explain the presence of Anatoliaca individuals (presumably from the Tuz Lake Basin) in Seydişehir and Caralitana individuals (presumably from the Konya Basin) just south Tuz Lake.

The occurence of orange-brown colored maculation, which can be considered as a intermediate character between *caralitana* and other water frog forms, usually coincide with regions where introgression was detected between Caralitana and Anatoliaca groups, hence pointing to probable hybrid individuals. Although not given in this thesis, results based on nuclear ITS2 marker indicate that gene flow among different water frog groups is even more widespread than shown by mtDNA findings (unpublished data).

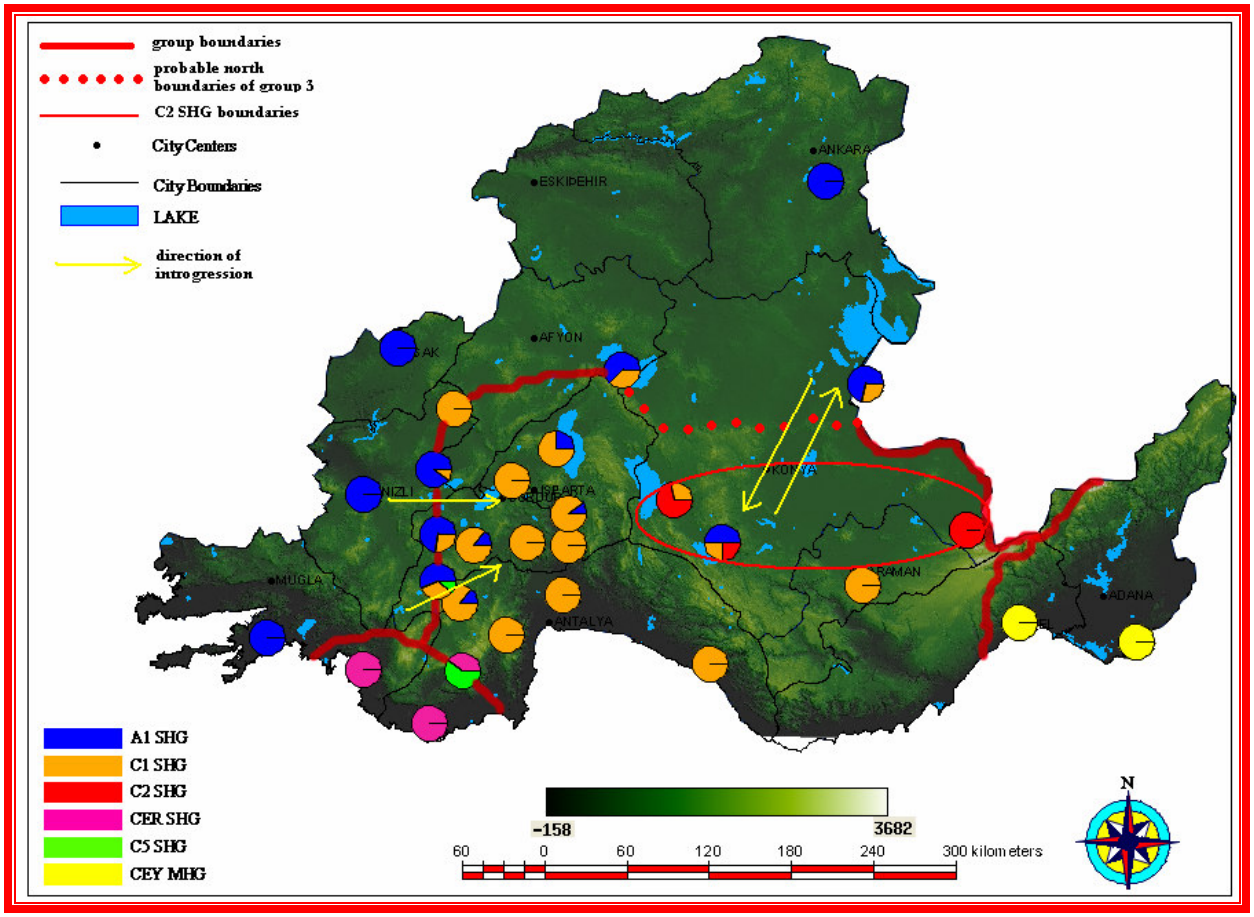


Figure 3-14. Map showing the direction of introgression and hybrid zone regions (Kaya, 2006).

3.5. Taxonomical Implications

Taking into account the genetic distance values and specific substitutions among /within MHGs, the genetic structure and relationships of MHGs within Minimum Spanning Network and a significant geographic structuring of groups of populations, it is proposed that there are 4 distinct water frog MHGs in Turkey: Caralitana MHG, Anatoliaca MHG (including Cerigensis), Ceyhanensis MHG and Ridibunda MHG.

These results support both Plötner et al. (2001) who used similar molecular markers and Jdeidi et al. (2001) who used morphometric, allozyme and bioacoustic parameters. Jdeidi et al. (2001) had shown that water frogs in Turkey are divided into 3 groups: The first group included *Rana ridibunda* in European Turkey, the second group included *R. caralitana* of the Lake District and Konya plain, which they proposed to raise to species status, and the last one had *R. bedriagae* in the rest of Anatolia. Nevertheless, both Plötner et al. (2001) and our findings indicate that the true *R. bedriagae* clade is clearly different from the clade in most of Asiatic Turkey (i.e. our Anatoliaca MHG) and genetic distance between them range from 0.036 to 0.052. Thus, we confirm that no Turkish water frog populations belong to *R. bedriagae*.

There is no consensus on how many or which water frog species occur in Turkey. The recent treatment of Turkish water frog diversity ranges from just a single species (*ridibunda*; Özeti and Yılmaz, 1994; Baran, 2005) to two species (*ridibunda* and *levantina* (=*bedriagae*); Baran and Atatür 1998) and up to three species (*ridibunda*, *caralitana*, *bedriagae*; Anonymous, 2006; Frost, 2006). The extraordinary color and pattern variation observed within any population, as well as the lack of obvious morphological/morphometric diagnostic characters to differentiate between geographic populations may explain the conservative attitude of many anuran taxonomists. However, amphibans in general, and

water frogs in particular, are known to contain many cryptic species (Köhler et al., 2005; Plötner, 2005). Geographically well structured populations with considerable differences in mtDNA sequences are generally accepted to represent separate species.

Our results confirm previous work by others that *R. ridibunda sensu stricto* occurs only in European Turkey. This lineage is quite divergent from other Turkish populations and has diagnostic allozyme combinations and bioacoustical properties (Jdeidi et al., 1999; 2001).

Another highly divergent clade occurs exclusively on the Cilician plain, i.e. in Adana and (eastern) İçel provinces. Its status as a unique but geographically restricted lineage has already been suggested by Plötner & Ohst (2001), and our data (with the help of a higher sample size and additional localities) have confirmed this. These populations that carry haplotypes exclusively of the Ceyhanensis type apparently belong to a yet unnamed taxon. However, the eastern border of this taxon's range is not clearly set and should be investigated.

The water frogs that occur in the rest of Turkey have been shown to be neither *R. ridibunda sensu stricto* nor *R. bedriagae*. The taxonomic identity of these populations are rather complicated. In 1994, Beerli et al. have described a new species, *Rana cerigensis,* from Karpathos and Rhodes (Greece). Our results show that the typical Cerigensis haplotypes are found in populations from the coastal parts of southwestern Turkey, opposite to those Greek islands. Turkish frogs of that lineage are more diverse than Karpathos and Rhodes frogs. Karpathos & Rhodes islands were connected to the Anatolian mainland 3-5 million years ago (Plötner & Ohst, 2001). All these point to an Anatolian origin of *R. cerigensis* and the very small difference between Turkish and Greek haplotypes warrants an expansion of its range to southwestern Turkey.

However, the relatively small degree of difference between Cerigensis and Anatoliaca haplotypes as well as the adjacency of their ranges question the validity of restricting *R. cerigensis* to southwestern Turkey and the opposite Greek islands. According to the nomenclatural rules, the name *cerigensis* should then include populations carrying haplotypes of the Anatoliaca-type. The more or less clearly separated geographic ranges of each SHGs within MHGs may be treated as distinct subspecies. Hence, if *R. cerigensis* is a valid species, it should consist of two subspecies: The nominate subspecies in west coastal part of Antalya, east coastal part of Muğla, Karpathos and Rhodes, and subspecies *anatoliaca* in the rest of the species' range.

The taxon *caralitana* had been described by Arıkan as a subspecies of *R. ridibunda* in 1988. The unique and moderately divergent maternal lineage of Caralitana (including C1, C2 and C5 subhaplogroups), the presence of orange spotted venter color (albeit irregularly), diagnostic morphometric, allozyme and bioacoustic characters (Jdeidi et al., 2001), and chromosomal differences from surrounding forms (Alpagut and Falakalı, 2006) all suggest *caralitana* is a species of its own.

The only problem, however, is the degree of apparent hybridization between this form and the Anatoliaca- or Cerigensis-type individuals near the border of their respective ranges. Since the level and nature of this hybridization is not wholly clear, it is difficult to assess the degree of reproductive isolation. If it can be conclusively shown that there exists considerable isolating barriers between Lake District/Konya populations and their neighbors, then the presence of two separate species - *R. caralitana* and *R. cerigensis* - in western Turkey is granted.

If, on the other hand, these forms freely interbreed despite the occurence of divergent characters listed above, then they should instead be considered separate subspecies of the same species. That would make all individuals that

carry Cerigensis, Anatoliaca or Caralitana haplotypes conspecific. According to the rule of priority, the name *Rana caralitana* should be used to refer to them all.

Based on the findings of this study, water frog taxa that should be listed as part of the Turkish fauna are given below, assuming that the observed hybridization is strong enough to lead to considerable genetic exchange between Lake District/Konya frogs and neighboring populations (Table 3-6). Alternatively, if *R. caralitana* is restricted to Lake District/Konya populations only, then *R. cerigensis* (representing populations from most of the rest of Turkey, Karpathos-Rhodes and northeastern Syria) should be added as a fourth species.

Table 3-6. Rana (Pelophylax) species in Turkey in 2007.

Rana ridibunda Pallas 1771	
Range:	European Turkey
Rana caralitana Arıkan 1988	
Range:	Asiatic Turkey (except for Cilicia), Rhodes & Karpathos, northeastern Syria; probably also Iraq and Transcaucasia.
Subspp:	*caralitana* (Anatolian Lakes District, Antalya, Konya and Karaman)
	cerigensis (coastal Muğla and western Antalya, Rhodes, Karpathos)
	anatoliaca subspp. nov. (rest of the species' range)
Rana ceyhanensis spp. nov.	
Range:	Cilicia plain (İçel, Adana and Osmaniye; possibly Hatay

However, to reveal the extent of gene flow or the degree of reproductive isolation among different species and subspecies, not only nuclear markers but also crossing and mate choice experiments are needed.

Divergence times of Anatolian water frog groups were estimated using the correlation based on the genetic distance of 0.072 between *Rana cretensis* and individuals from mainland and a 5.2 myr divergence time of Crete (Plötner et al., 2001). Based on this calibration, approximate divergence time between *Rana caralitana* and *R. cerigensis* is 1.3 - 3.5 myr, between *R. caralitana* and *R. ceyhanensis* 2.8 - 4.2 myr, and between *R. cerigensis* and *R. ceyhanensis* 2.4 - 3.5 myr. Also, divergence time of sublineages within *R. cerigensis* is about 0.87 - 2.2 myr; similarly within *R. caralitana* it is 0.87 - 2.0 myr. All species have probably differentiated in late Pliocene and early Pleistocene and (around the Neogene and Quaternary boundary). Following the last Quaternary glaciation period, water frogs groups seem to have expanded from Lycian (*sensu* Schmidtler, 1998), Pamphylian and Cilician refugias to the rest of Anatolia.

The Lycian Pleistocene refuge is well known (Schmidtler, 1998). The ranges of several endemic taxa such as *Lyciasalamandra luschani*, *L. fazilae* and *Ophiomorus punctatissimus* are currently restricted to the refuge but several taxa now much more widespread are accepted to have sprang from that refuge in the past (e.g. mountain frogs, Veith et al., 2003).

Pamphylia (i.e. the lowlands of the Antalya province) has not been proposed as a Pleistocene refuge before although there are several (endemic) species that are restricted to Antalya (and sometimes spreading north into Lake District). These include *Lyciasalamandra atifi, L. antalyana*, *L. billae*, *Pseudophoxinus antalyae*, *Capoeta antalyensis* and *Lacerta pamphylica.* The exclusive presence of several fish species in both Lake District and the Konya plain also point to a common source, most probably the Pamphylian refuge, to (re)colonize aquatic habitats

there. The endemics *Pseudophoxinus anatolicus*, *P. battalgili*, *P. fahirae* and *Cobitis turcica* have ranges that link the current range of Caralitana-type populations (Cox et al., 2006, Smith and Darwall, 2006).

The Cilician refuge (sometimes called Bolkar refuge, Schmidtler, 1998) seems to have given rise to taxa such as *Salamandra infraimmaculata orientalis*, *Triturus vittatus cilicensis* and *Eirenis barani* (Cox et al., 2006; Smith and Darwall, 2006).

The observed zoogeographical patterns for *Rana* are also largely paralleled by those obtained through molecular studies for other aquatic species complexes. For instance, using 3434 bp of mtDNA gene regions, including ND1, ND2, 12S, 16S rRNAs and many tRNA genes, three clear divergent *Aphanius anatoliae* (Pisces, Cyprinodontiformes) clades were shown to occupy the Tuz Gölü Basin, Lakes District and Southwest Bey Mountain regions (Hrbek et al., 2002). Similarly, using complete cyt-*b* gene region of mtDNA, three deeply divergent *Pseudophoxinus* (Pisces: Cypriniformes) species (*P. anatolicus* on the Tuz Gölü Basin, *P. meandri* on the Lakes District and *P. fahirae* in the soutwest) were revealed (Hrbek et al., 2004). These results are generally concordant with the geographic distribution of C1 SHG on the Lakes District Region, C2 SHG on the Konya plain and Cer SHG in the southwestern region, respectively.

CHAPTER 4

CONCLUSIONS

✹ Results of mtDNA ND3 sequences show considerable diversity and strong geographical structure for water frog populations on the Southwestern part of Turkey.

✹ There are four mainhaplogroups in Turkey, each of which probably represents a separate taxon (probably species).

1. **Ridibunda MHG:** This MHG occurs only in European part of Turkey (Thrace) and represents *Rana ridibunda* Pallas 1771 sensu stricto.

2. **Ceyhanensis MHG:** A highly divergent lineage that occurs exclusively in İçel, Adana and Osmaniye provinces (Cilicia plain). This is an unnamed taxon and the eastern borders of its range is yet to be determined.

3. **Caralitana MHG:** This is a distinct lineage with specific diverse haplotypes, usually an orange spotted venter, and diagnostic morphometric and bioacoustic characters. Its range includes Anatolian Lake District, Antalya, Konya & Karaman provinces. It has three sublineages, the widespread C1 SHG and two other more restricted SHGs (C2 and C5). These populations are best represented by *Rana caralitana* taxon (Arıkan 1988). However, another

interpretation would expand the members of this taxon to most of Anatolia, except for Ceyhanensis-type populations.

4. **Anatoliaca MHG:** This is the most widespread MHG and particular haplotypes can be found at localities that are several hundred kilometers apart; other haplotypes (e.g. A3) are more localized. These populations do not belong to *R. bedriagae* but instead should be considered as subspecies of either *R. caralitana* or *R. cerigensis*. *R. cerigensis* Beerli, Hotz, Tunner, Heppich, and Uzzell 1994 was originally described as a new species from Rhodes and Karpathos. The haplotypes typical for *cerigensis* are not particularly different than other haplotypes of the Anatoliaca MHG, therefore, according to the priority rule Anatoliaca MHG should be considered as *Rana cerigensis,* which is the oldest available and a valid name for this taxon. Thus, its range includes Asiatic Turkey (except for central southern Turkey), Rhodes & Karpathos, northeastern Syria, and probably also Iraq and Transcaucasia.

✹ Caralitana lineage and unnamed Anatolian lineages are largely parapatric and reciprocal introgression is clearly present between them. Introgression especially in western borders of Lake District, intermediate individuals in terms of venter color overlap considerably with populations where haplotype from different MHGs co-exist.

✹ The orange venter color is not always associated with caralitana mtDNA haplotypes. Therefore, it is not reliable by itself alone to diagnose any *caralitana* form and combination of other characters is required for taxonomic characterization and identification.

✹ The observed zoogeographical patterns suggest that each of the Anatolian main haplogroups had diverged during the Pleistocene at separate refuges: the

Anatoliaca/Cerigensis lineage at the Lycian refuge, the Caralitana lineage at the Pamphylian refuge, and the Ceyhan lineage at the Cilician refuge.

✹ These results indicate the existence of many cryptic water frog groups on the Southwestern part of Turkey, which are morphologically similar and otherwise indistinguishable from each other.

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APPENDIX A: Table of Pairwise Distance

Table A. Jukes- Cantor distances (below diagonal) and numbers of substitutions (transitions/transversions/amino acids) between the mt DNA's haplotypes of the water frogs forms (above the diagonal).

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	**Caralitana1-10**		1 / 1 / 1	0 / 1 / 0	6 / 4 / 2	1 / 1 / 1	1 / 0 / 0	2 / 1 / 1	8 / 6 / 3	8 / 6 / 3	9 / 6 / 3	9 / 6 / 3	9 / 6 / 3	1 / 0 / 1
2	**Caralitana3-11**	0.006		1 / 0 / 1	7 / 3 / 3	2 / 0 / 2	2 / 1 / 1	3 / 0 / 2	9 / 5 / 4	9 / 5 / 4	10 / 5 / 4	10 / 5 / 4	10 / 5 / 4	2 / 1 / 2
3	**Caralitana3-10**	0.003	0.003		6 / 3 / 2	1 / 0 / 1	1 / 1 / 0	2 / 0 / 1	8 / 5 / 3	8 / 5 / 3	9 / 5 / 3	9 / 5 / 3	9 / 5 / 3	1 / 1 / 1
4	**Anatoliaca2-10**	0.030	0.030	0.027		7 / 3 / 3	6 / 4 / 2	8 / 3 / 3	12 / 2 / 1	12 / 2 / 1	13 / 2 / 1	13 / 2 / 1	13 / 2 / 1	6 / 4 / 2
5	**Caralitana4-10**	0.006	0.006	0.003	0.030		2 / 1 / 1	1 / 0 / 0	9 / 5 / 4	9 / 5 / 4	10 / 5 / 4	10 / 5 / 4	10 / 5 / 4	2 / 1 / 2
6	**Caralitana1-11**	0.003	0.009	0.006	0.030	0.009		3 / 1 / 1	8 / 6 / 3	8 / 6 / 3	9 / 6 / 3	9 / 6 / 3	9 / 6 / 3	2 / 0 / 1
7	**Caralitana4-11**	0.009	0.009	0.006	0.033	0.003	0.012		10 / 5 / 4	10 / 5 / 4	11 / 5 / 4	11 / 5 / 4	11 / 5 / 4	3 / 1 / 2
8	**Ceyhanensis1-10**	0.042	0.042	0.039	0.042	0.042	0.042	0.045		2 / 0 / 0	3 / 0 / 0	3 / 0 / 0	1 / 0 / 0	8 / 6 / 3
9	**Ceyhanensis1-21**	0.042	0.042	0.039	0.042	0.042	0.042	0.045	0.006		1 / 0 / 0	3 / 0 / 0	1 / 0 / 0	8 / 6 / 3
10	**Ceyhanensis1-31**	0.045	0.045	0.042	0.045	0.045	0.045	0.049	0.009	0.003		4 / 0 / 0	2 / 0 / 0	9 / 6 / 3
11	**Ceyhanensis1-32**	0.045	0.045	0.042	0.045	0.045	0.045	0.049	0.009	0.009	0.012		2 / 0 / 0	9 / 6 / 3
12	**Ceyhanensis1-11**	0.045	0.045	0.042	0.045	0.045	0.045	0.049	0.003	0.003	0.006	0.006		9 / 6 / 3
13	**Caralitana1-12**	0.003	0.009	0.006	0.030	0.009	0.006	0.012	0.042	0.042	0.045	0.045	0.045	
14	**Caralitana1-13**	0.003	0.009	0.006	0.033	0.009	0.006	0.012	0.045	0.045	0.049	0.049	0.049	0.006
15	**Caralitana1-14**	0.003	0.009	0.006	0.033	0.009	0.006	0.012	0.045	0.045	0.049	0.042	0.049	0.006
16	**Caralitana1-21**	0.006	0.012	0.009	0.036	0.012	0.009	0.015	0.049	0.049	0.052	0.052	0.052	0.009
17	**Caralitana1-15**	0.003	0.009	0.006	0.027	0.009	0.006	0.012	0.045	0.045	0.049	0.049	0.049	0.006
18	**Caralitana1-16**	0.003	0.009	0.006	0.033	0.009	0.006	0.012	0.045	0.045	0.049	0.049	0.049	0.006
19	**Caralitana3-12**	0.003	0.009	0.006	0.033	0.003	0.006	0.006	0.045	0.045	0.049	0.049	0.049	0.006
20	**Anatoliaca2-11**	0.033	0.033	0.030	0.003	0.033	0.033	0.036	0.045	0.045	0.049	0.049	0.049	0.033
21	**Caralitana5-10**	0.018	0.018	0.015	0.036	0.012	0.021	0.015	0.055	0.055	0.058	0.058	0.058	0.021
22	**Anatoliaca1-10**	0.027	0.027	0.024	0.009	0.027	0.027	0.030	0.039	0.039	0.042	0.042	0.042	0.027
23	**Anatoliaca1-11**	0.024	0.024	0.021	0.006	0.024	0.024	0.027	0.036	0.036	0.039	0.039	0.039	0.024

Table A continued.

		1	2	3	4	5	6	7	8	9	10	11	12	13
24	**Ridibunda1-10**	0.074	0.074	0.071	0.068	0.074	0.074	0.077	0.055	0.061	0.061	0.064	0.058	0.074
25	**Anatoliaca1-21**	0.027	0.027	0.024	0.009	0.027	0.027	0.030	0.039	0.039	0.042	0.042	0.042	0.027
26	**Anatoliaca1-22**	0.021	0.021	0.018	0.009	0.021	0.021	0.024	0.033	0.033	0.036	0.036	0.036	0.021
27	**Anatoliaca1-23**	0.027	0.027	0.024	0.009	0.027	0.027	0.030	0.039	0.039	0.042	0.042	0.042	0.027
28	**Caralitana2-10**	0.021	0.021	0.018	0.042	0.015	0.018	0.018	0.055	0.055	0.058	0.058	0.058	0.024
29	**Caralitana2-21**	0.021	0.021	0.018	0.036	0.015	0.018	0.018	0.055	0.055	0.058	0.058	0.058	0.024
30	**Caralitana2-11**	0.018	0.018	0.015	0.039	0.012	0.015	0.015	0.052	0.052	0.055	0.055	0.055	0.021
31	**Caralitana1-17**	0.003	0.009	0.006	0.033	0.009	0.006	0.012	0.045	0.045	0.049	0.049	0.049	0.006
32	**Caralitana1-18**	0.003	0.009	0.006	0.033	0.009	0.006	0.012	0.045	0.045	0.049	0.049	0.049	0.006
33	**Anatoliaca3-10**	0.033	0.033	0.030	0.009	0.033	0.033	0.036	0.039	0.039	0.042	0.042	0.042	0.033
34	**Anatoliaca3-11**	0.030	0.030	0.027	0.006	0.030	0.030	0.033	0.042	0.042	0.045	0.045	0.045	0.030
35	**Anatoliaca3-12**	0.036	0.036	0.033	0.012	0.036	0.036	0.039	0.036	0.036	0.039	0.039	0.039	0.036
36	**Cerigensis1-11**	0.030	0.030	0.027	0.021	0.030	0.030	0.033	0.039	0.039	0.042	0.042	0.042	0.030
37	**Cerigensis1-12**	0.033	0.033	0.030	0.021	0.033	0.033	0.036	0.039	0.039	0.042	0.042	0.042	0.033
38	**Cerigensis1-21**	0.036	0.036	0.033	0.024	0.036	0.036	0.039	0.042	0.042	0.045	0.045	0.045	0.036
39	**Cerigensis1-10**	0.030	0.030	0.027	0.018	0.030	0.030	0.033	0.036	0.036	0.039	0.039	0.039	0.030
40	**Trodos Cyprus**	0.068	0.068	0.064	0.055	0.068	0.068	0.071	0.052	0.052	0.055	0.049	0.055	0.068
41	**Rana cretensis**	0.153	0.153	0.149	0.135	0.146	0.153	0.149	0.121	0.121	0.124	0.111	0.118	0.153
42	**Rana epeirotica**	0.149	0.153	0.149	0.146	0.146	0.149	0.149	0.153	0.146	0.149	0.146	0.149	0.149
43	**Rana lessonae**	0.164	0.160	0.164	0.153	0.167	0.164	0.171	0.146	0.146	0.149	0.149	0.149	0.164
44	**Syria Abu Kemal 313**	0.039	0.039	0.036	0.027	0.033	0.039	0.036	0.052	0.052	0.049	0.055	0.055	0.039
45	**Rana bedriagae 320**	0.049	0.049	0.045	0.042	0.049	0.049	0.052	0.042	0.042	0.045	0.039	0.045	0.049
46	**Rana bedriagae 322**	0.049	0.049	0.045	0.042	0.049	0.049	0.052	0.042	0.042	0.045	0.039	0.045	0.049
47	**Syria Abu Kemal 312**	0.036	0.036	0.033	0.024	0.030	0.036	0.033	0.049	0.049	0.052	0.052	0.052	0.036

Table A continued.

		14	15	16	17	18	19	20	21	22	23	24	25	26
1	**Caralitana1-10**	1 / 0 / 1	1 / 0 / 0	2 / 0 / 0	1 / 0 / 0	1 / 0 / 1	1 / 0 / 1	7 / 4 / 2	5 / 1 / 2	5 / 4 / 3	4 / 4 / 2	17 / 7 / 3	5 / 4 / 2	3 / 4 / 2
2	**Caralitana3-11**	2 / 1 / 2	2 / 1 / 1	3 / 1 / 1	2 / 1 / 1	2 / 1 / 2	2 / 1 / 2	8 / 3 / 3	6 / 0 / 3	6 / 3 / 4	5 / 3 / 3	18 / 6 / 4	6 / 3 / 3	4 / 3 / 3
3	**Caralitana3-10**	1 / 1 / 1	1 / 1 / 0	2 / 1 / 0	1 / 1 / 0	1 / 1 / 1	1 / 1 / 1	7 / 3 / 2	5 / 0 / 2	5 / 3 / 3	4 / 3 / 2	17 / 6 / 3	5 / 3 / 2	3 / 3 / 2
4	**Anatoliaca2-10**	7 / 4 / 3	7 / 4 / 2	8 / 4 / 2	5 / 4 / 2	7 / 4 / 3	7 / 4 / 3	1 / 0 / 0	9 / 3 / 4	3 / 0 / 1	2 / 0 / 0	19 / 3 / 1	3 / 0 / 0	3 / 0 / 0
5	**Caralitana4-10**	2 / 1 / 2	2 / 1 / 1	3 / 1 / 1	2 / 1 / 1	2 / 1 / 2	0 / 1 / 0	8 / 3 / 3	4 / 0 / 1	6 / 3 / 4	5 / 3 / 3	18 / 6 / 4	6 / 3 / 3	4 / 3 / 3
6	**Caralitana1-11**	2 / 0 / 1	2 / 0 / 0	3 / 0 / 0	2 / 0 / 0	2 / 0 / 1	2 / 0 / 1	7 / 4 / 2	6 / 1 / 2	5 / 4 / 3	4 / 4 / 2	17 / 7 / 3	5 / 4 / 2	3 / 4 / 2
7	**Caralitana4-11**	3 / 1 / 2	3 / 1 / 1	4 / 1 / 1	3 / 1 / 1	3 / 1 / 2	1 / 1 / 0	9 / 3 / 3	5 / 0 / 1	7 / 3 / 4	6 / 3 / 3	19 / 6 / 4	7 / 3 / 3	5 / 3 / 3
8	**Ceyhanensis1-10**	9 / 6 / 4	9 / 6 / 3	10 / 6 / 3	9 / 6 / 3	9 / 6 / 4	9 / 6 / 4	13 / 2 / 1	13 / 5 / 5	11 / 2 / 2	10 / 2 / 1	15 / 3 / 2	11 / 2 / 1	9 / 2 / 1
9	**Ceyhanensis1-21**	9 / 6 / 4	9 / 6 / 3	10 / 6 / 3	9 / 6 / 3	9 / 6 / 4	9 / 6 / 4	13 / 2 / 1	13 / 5 / 5	11 / 2 / 2	10 / 2 / 1	17 / 3 / 2	11 / 2 / 1	9 / 2 / 1
10	**Ceyhanensis1-31**	10 / 6 / 4	10 / 6 / 3	11 / 6 / 3	10 / 6 / 3	10 / 6 / 4	10 / 6 / 4	14 / 2 / 1	14 / 5 / 5	12 / 2 / 2	11 / 2 / 1	17 / 3 / 2	12 / 2 / 1	10 / 2 / 1
11	**Ceyhanensis1-32**	10 / 6 / 4	8 / 6 / 3	11 / 6 / 3	10 / 6 / 3	10 / 6 / 4	10 / 6 / 4	14 / 2 / 1	14 / 5 / 5	12 / 2 / 2	11 / 2 / 1	18 / 3 / 2	12 / 2 / 1	10 / 2 / 1
12	**Ceyhanensis1-11**	10 / 6 / 4	10 / 6 / 3	11 / 6 / 3	10 / 6 / 3	10 / 6 / 4	10 / 6 / 4	14 / 2 / 1	14 / 5 / 5	12 / 2 / 2	11 / 2 / 1	16 / 3 / 2	12 / 2 / 1	10 / 2 / 1
13	**Caralitana1-12**	2 / 0 / 2	2 / 0 / 1	3 / 0 / 1	2 / 0 / 1	2 / 0 / 2	2 / 0 / 2	7 / 4 / 2	6 / 1 / 3	5 / 4 / 3	4 / 4 / 2	17 / 7 / 3	5 / 4 / 2	3 / 4 / 2
14	**Caralitana1-13**		2 / 0 / 1	3 / 0 / 1	2 / 0 / 1	2 / 0 / 2	2 / 0 / 2	8 / 4 / 3	6 / 1 / 3	6 / 4 / 4	5 / 4 / 3	18 / 7 / 4	6 / 4 / 3	4 / 4 / 3
15	**Caralitana1-14**	0.006		3 / 0 / 0	2 / 0 / 0	2 / 0 / 1	2 / 0 / 1	8 / 4 / 2	6 / 1 / 2	6 / 4 / 3	5 / 4 / 2	18 / 7 / 3	6 / 4 / 2	4 / 4 / 2
16	**Caralitana1-21**	0.009	0.009		3 / 0 / 0	3 / 0 / 1	3 / 0 / 1	9 / 4 / 2	7 / 4 / 2	7 / 4 / 3	6 / 4 / 2	17 / 7 / 3	7 / 4 / 2	5 / 4 / 2
17	**Caralitana1-15**	0.006	0.006	0.009		2 / 0 / 1	2 / 0 / 1	6 / 4 / 2	4 / 1 / 2	6 / 4 / 3	5 / 4 / 2	18 / 7 / 3	6 / 4 / 2	4 / 4 / 2
18	**Caralitana1-16**	0.006	0.006	0.009	0.006		2 / 0 / 2	8 / 4 / 3	6 / 1 / 3	6 / 4 / 4	5 / 4 / 3	18 / 7 / 4	6 / 4 / 3	4 / 4 / 3
19	**Caralitana3-12**	0.006	0.006	0.009	0.006	0.006		8 / 4 / 3	4 / 1 / 1	6 / 4 / 4	5 / 4 / 3	18 / 7 / 4	6 / 4 / 3	4 / 4 / 3
20	**Anatoliaca2-11**	0.036	0.036	0.039	0.030	0.036	0.036		10 / 3 / 4	4 / 0 / 1	3 / 0 / 0	20 / 3 / 1	4 / 0 / 0	4 / 0 / 0
21	**Caralitana5-10**	0.021	0.021	0.024	0.015	0.021	0.015	0.039		10 / 3 / 5	9 / 3 / 4	20 / 6 / 5	10 / 3 / 4	8 / 3 / 4
22	**Anatoliaca1-10**	0.030	0.030	0.033	0.030	0.030	0.030	0.012	0.039		1 / 0 / 1	18 / 3 / 2	2 / 0 / 1	2 / 0 / 1
23	**Anatoliaca1-11**	0.027	0.027	0.030	0.027	0.027	0.027	0.009	0.036	0.003		17 / 3 / 1	1 / 0 / 0	1 / 0 / 0

Table A continued.

		14	15	16	17	18	19	20	21	22	23	24	25	26
24	**Ridibunda1-10**	0.077	0.077	0.074	0.077	0.077	0.077	0.071	0.081	0.064	0.061		18 / 3 / 1	16 / 3 / 1
25	**Anatoliaca1-21**	0.030	0.030	0.033	0.030	0.030	0.030	0.012	0.039	0.006	0.003	0.064		2 / 0 / 0
26	**Anatoliaca1-22**	0.024	0.024	0.027	0.024	0.024	0.024	0.012	0.033	0.006	0.003	0.058	0.006	
27	**Anatoliaca1-23**	0.030	0.030	0.033	0.030	0.030	0.030	0.012	0.039	0.006	0.003	0.064	0.006	0.006
28	**Caralitana2-10**	0.024	0.024	0.027	0.024	0.024	0.018	0.045	0.027	0.039	0.036	0.087	0.039	0.033
29	**Caralitana2-21**	0.024	0.024	0.027	0.024	0.024	0.018	0.039	0.027	0.033	0.030	0.081	0.033	0.027
30	**Caralitana2-11**	0.021	0.021	0.024	0.021	0.021	0.015	0.042	0.024	0.036	0.033	0.084	0.036	0.030
31	**Caralitana1-17**	0.006	0.006	0.009	0.006	0.006	0.006	0.036	0.021	0.030	0.027	0.077	0.030	0.024
32	**Caralitana1-18**	0.006	0.006	0.009	0.006	0.006	0.006	0.036	0.021	0.030	0.027	0.077	0.030	0.024
33	**Anatoliaca3-10**	0.036	0.036	0.039	0.030	0.036	0.036	0.012	0.039	0.012	0.009	0.071	0.012	0.012
34	**Anatoliaca3-11**	0.033	0.033	0.036	0.027	0.033	0.033	0.009	0.036	0.009	0.006	0.068	0.009	0.009
35	**Anatoliaca3-12**	0.039	0.039	0.042	0.033	0.039	0.039	0.015	0.042	0.015	0.012	0.068	0.015	0.015
36	**Cerigensis1-11**	0.033	0.033	0.036	0.033	0.033	0.033	0.024	0.042	0.018	0.015	0.064	0.018	0.018
37	**Cerigensis1-12**	0.036	0.036	0.039	0.036	0.036	0.036	0.024	0.045	0.018	0.015	0.064	0.018	0.018
38	**Cerigensis1-21**	0.039	0.039	0.042	0.039	0.039	0.039	0.027	0.049	0.021	0.018	0.068	0.021	0.021
39	**Cerigensis1-10**	0.033	0.033	0.036	0.033	0.033	0.033	0.021	0.042	0.015	0.012	0.061	0.015	0.015
40	**Trodos Cyprus**	0.071	0.064	0.074	0.064	0.071	0.071	0.058	0.074	0.064	0.061	0.094	0.058	0.058
41	**Rana cretensis**	0.156	0.149	0.160	0.149	0.156	0.149	0.139	0.142	0.139	0.135	0.131	0.139	0.131
42	**Rana epeirotica**	0.153	0.146	0.149	0.146	0.153	0.146	0.149	0.146	0.156	0.153	0.142	0.156	0.149
43	**Rana lessonae**	0.167	0.160	0.156	0.160	0.167	0.167	0.149	0.164	0.164	0.160	0.171	0.164	0.156
44	**Syria Abu Kemal 313**	0.042	0.042	0.045	0.042	0.042	0.036	0.030	0.045	0.024	0.021	0.074	0.024	0.024
45	**Rana bedriagae 320**	0.052	0.045	0.055	0.052	0.052	0.052	0.045	0.061	0.045	0.042	0.061	0.045	0.039
46	**Rana bedriagae 322**	0.052	0.045	0.055	0.052	0.052	0.052	0.045	0.061	0.045	0.042	0.061	0.045	0.039
47	**Syria Abu Kemal 312**	0.039	0.039	0.042	0.039	0.039	0.033	0.027	0.042	0.021	0.018	0.081	0.015	0.021

Table A conitnued.

		27	28	29	30	31	32	33	34	35	36	37	38	39
1	**Caralitana1-10**	5 / 4 / 2	5 / 2 / 2	5 / 2 / 2	4 / 2 / 2	0 / 1 / 1	1 / 0 / 1	7 / 4 / 3	6 / 4 / 3	8 / 4 / 3	6 / 4 / 3	7 / 4 / 3	8 / 4 / 3	6 / 4 / 3
2	**Caralitana3-11**	6 / 3 / 3	6 / 1 / 3	6 / 1 / 3	5 / 1 / 3	1 / 2 / 1	2 / 1 / 2	8 / 3 / 4	7 / 3 / 4	9 / 3 / 4	7 / 3 / 4	8 / 3 / 4	9 / 3 / 4	7 / 3 / 4
3	**Caralitana3-10**	5 / 3 / 2	5 / 1 / 2	5 / 1 / 2	4 / 1 / 2	0 / 2 / 0	1 / 1 / 1	7 / 3 / 3	6 / 3 / 3	8 / 3 / 3	6 / 3 / 3	7 / 3 / 3	8 / 3 / 3	6 / 3 / 3
4	**Anatoliaca2-10**	3 / 0 / 0	10 / 4 / 4	8 / 4 / 4	9 / 4 / 4	6 / 5 / 2	7 / 4 / 3	3 / 0 / 1	2 / 0 / 1	4 / 0 / 1	7 / 0 / 2	7 / 0 / 1	8 / 0 / 1	6 / 0 / 1
5	**Caralitana4-10**	6 / 3 / 3	4 / 1 / 1	4 / 1 / 1	3 / 1 / 1	1 / 2 / 1	2 / 1 / 2	8 / 3 / 4	7 / 3 / 4	9 / 3 / 4	7 / 3 / 4	8 / 3 / 4	9 / 3 / 4	7 / 3 / 4
6	**Caralitana1-11**	5 / 4 / 2	4 / 2 / 2	4 / 2 / 2	3 / 2 / 2	1 / 1 / 0	2 / 0 / 1	7 / 4 / 3	6 / 4 / 3	8 / 4 / 3	6 / 4 / 3	7 / 4 / 3	8 / 4 / 3	6 / 4 / 3
7	**Caralitana4-11**	7 / 3 / 3	5 / 1 / 1	5 / 1 / 1	4 / 1 / 1	2 / 2 / 1	3 / 1 / 2	9 / 3 / 4	8 / 3 / 4	10 / 3 / 4	8 / 3 / 4	9 / 3 / 4	10 / 3 / 4	8 / 3 / 4
8	**Ceyhanensis1-10**	11 / 2 / 1	12 / 6 / 5	12 / 6 / 5	11 / 6 / 5	8 / 7 / 3	9 / 6 / 4	11 / 2 / 2	12 / 2 / 2	10 / 2 / 2	11 / 2 / 3	11 / 2 / 2	12 / 2 / 2	10 / 2 / 2
9	**Ceyhanensis1-21**	11 / 2 / 1	12 / 6 / 5	12 / 6 / 5	11 / 6 / 5	8 / 7 / 3	9 / 6 / 4	11 / 2 / 2	12 / 2 / 2	10 / 2 / 2	11 / 2 / 3	11 / 2 / 2	12 / 2 / 2	10 / 2 / 2
10	**Ceyhanensis1-31**	12 / 2 / 1	13 / 6 / 5	13 / 6 / 5	12 / 6 / 5	9 / 7 / 3	10 / 6 / 4	12 / 2 / 2	13 / 2 / 2	11 / 2 / 2	12 / 2 / 3	12 / 2 / 2	13 / 2 / 2	11 / 2 / 2
11	**Ceyhanensis1-32**	12 / 2 / 1	13 / 6 / 5	13 / 6 / 5	12 / 6 / 5	9 / 7 / 3	10 / 6 / 4	12 / 2 / 2	13 / 2 / 2	11 / 2 / 2	12 / 2 / 3	12 / 2 / 2	13 / 2 / 2	11 / 2 / 2
12	**Ceyhanensis1-11**	12 / 2 / 1	13 / 6 / 5	13 / 6 / 5	12 / 6 / 5	9 / 7 / 3	10 / 6 / 4	12 / 2 / 2	13 / 2 / 2	11 / 2 / 2	12 / 2 / 3	12 / 2 / 2	13 / 2 / 2	11 / 2 / 2
13	**Caralitana1-12**	5 / 4 / 2	6 / 2 / 3	6 / 2 / 3	5 / 2 / 3	1 / 1 / 1	2 / 0 / 2	7 / 4 / 3	6 / 4 / 3	8 / 4 / 3	6 / 4 / 3	7 / 4 / 3	8 / 4 / 3	6 / 4 / 3
14	**Caralitana1-13**	6 / 4 / 3	6 / 2 / 3	6 / 2 / 3	5 / 2 / 3	1 / 1 / 1	2 / 0 / 2	8 / 4 / 4	7 / 4 / 4	9 / 4 / 4	7 / 4 / 4	8 / 4 / 4	9 / 4 / 4	7 / 4 / 4
15	**Caralitana1-14**	6 / 4 / 2	6 / 2 / 2	6 / 2 / 2	5 / 2 / 2	1 / 1 / 0	2 / 0 / 1	8 / 4 / 3	7 / 4 / 3	9 / 4 / 3	7 / 4 / 3	8 / 4 / 3	9 / 4 / 3	7 / 4 / 3
16	**Caralitana1-21**	7 / 4 / 2	7 / 2 / 2	7 / 2 / 2	6 / 2 / 2	2 / 1 / 0	3 / 0 / 1	9 / 4 / 3	8 / 4 / 3	10 / 4 / 3	8 / 4 / 3	9 / 4 / 3	10 / 4 / 3	8 / 4 / 3
17	**Caralitana1-15**	6 / 4 / 2	6 / 2 / 2	6 / 2 / 2	5 / 2 / 2	1 / 1 / 0	2 / 0 / 1	6 / 4 / 3	5 / 4 / 3	7 / 4 / 3	7 / 4 / 3	8 / 4 / 3	9 / 4 / 3	7 / 4 / 3
18	**Caralitana1-16**	6 / 4 / 3	6 / 2 / 3	6 / 2 / 3	5 / 2 / 3	1 / 1 / 1	2 / 0 / 2	8 / 4 / 4	7 / 4 / 4	9 / 4 / 4	7 / 4 / 4	8 / 4 / 4	9 / 4 / 4	7 / 4 / 4
19	**Caralitana3-12**	6 / 4 / 3	4 / 2 / 1	4 / 2 / 1	3 / 2 / 1	1 / 1 / 1	2 / 0 / 2	8 / 4 / 4	7 / 4 / 4	9 / 4 / 4	7 / 4 / 4	8 / 4 / 4	9 / 4 / 4	7 / 4 / 4
20	**Anatoliaca2-11**	4 / 0 / 0	11 / 4 / 4	9 / 4 / 4	10 / 4 / 4	7 / 5 / 2	8 / 4 / 3	4 / 0 / 1	3 / 0 / 1	5 / 0 / 1	8 / 0 / 2	8 / 0 / 1	9 / 0 / 1	7 / 0 / 1
21	**Caralitana5-10**	10 / 3 / 4	8 / 1 / 2	8 / 1 / 2	7 / 1 / 2	5 / 2 / 2	6 / 1 / 3	10 / 3 / 5	9 / 3 / 5	11 / 3 / 5	11 / 3 / 5	12 / 3 / 5	13 / 3 / 5	11 / 3 / 5
22	**Anatoliaca1-10**	2 / 0 / 1	9 / 4 / 5	7 / 4 / 5	8 / 4 / 5	5 / 5 / 3	6 / 4 / 4	4 / 0 / 2	3 / 0 / 2	5 / 0 / 2	6 / 0 / 3	6 / 0 / 2	7 / 0 / 2	5 / 0 / 2
23	**Anatoliaca1-11**	1 / 0 / 0	8 / 4 / 4	6 / 4 / 4	7 / 4 / 4	4 / 5 / 2	5 / 4 / 3	3 / 0 / 1	2 / 0 / 1	4 / 0 / 1	5 / 0 / 2	5 / 0 / 1	6 / 0 / 1	4 / 0 / 1

Table A continued.

		27	28	29	30	31	32	33	34	35	36	37	38	39
24	**Ridibunda1-10**	18 / 3 / 1	21 / 7 / 5	19 / 7 / 5	20 / 7 / 5	17 / 8 / 3	18 / 7 / 4	20 / 3 / 2	19 / 3 / 2	19 / 3 / 2	18 / 3 / 3	18 / 3 / 2	19 / 3 / 2	17 / 3 / 2
25	**Anatoliaca1-21**	2 / 0 / 0	9 / 4 / 4	7 / 4 / 4	8 / 4 / 4	5 / 5 / 2	6 / 4 / 3	4 / 0 / 1	3 / 0 / 1	5 / 0 / 1	6 / 0 / 2	6 / 0 / 1	7 / 0 / 1	5 / 0 / 1
26	**Anatoliaca1-22**	2 / 0 / 0	7 / 4 / 4	5 / 4 / 4	6 / 4 / 4	3 / 5 / 2	4 / 4 / 3	4 / 0 / 1	3 / 0 / 1	5 / 0 / 1	6 / 0 / 2	6 / 0 / 1	7 / 0 / 1	5 / 0 / 1
27	**Anatoliaca1-23**		7 / 4 / 4	5 / 4 / 4	6 / 4 / 4	5 / 5 / 2	6 / 4 / 3	4 / 0 / 1	3 / 0 / 1	5 / 0 / 1	6 / 0 / 2	6 / 0 / 1	7 / 0 / 1	5 / 0 / 1
28	**Caralitana2-10**	0.033		2 / 0 / 0	1 / 0 / 0	5 / 3 / 2	6 / 2 / 3	11 / 4 / 5	10 / 4 / 5	12 / 4 / 5	10 / 4 / 5	11 / 4 / 5	12 / 4 / 5	10 / 4 / 5
29	**Caralitana2-21**	0.027	0.006		1 / 0 / 0	5 / 3 / 2	6 / 2 / 3	9 / 4 / 5	8 / 4 / 5	10 / 4 / 5	10 / 4 / 5	11 / 4 / 5	12 / 4 / 5	10 / 4 / 5
30	**Caralitana2-11**	0.030	0.003	0.003		4 / 3 / 2	5 / 2 / 3	10 / 4 / 5	9 / 4 / 5	11 / 4 / 5	9 / 4 / 5	10 / 4 / 5	11 / 4 / 5	9 / 4 / 5
31	**Caralitana1-17**	0.030	0.024	0.024	0.021		1 / 1 / 1	7 / 5 / 3	6 / 5 / 3	8 / 5 / 3	6 / 5 / 3	7 / 5 / 3	8 / 5 / 3	6 / 5 / 3
32	**Caralitana1-18**	0.030	0.024	0.024	0.021	0.006		8 / 4 / 4	7 / 4 / 4	9 / 4 / 4	7 / 4 / 4	8 / 4 / 4	9 / 4 / 4	7 / 4 / 4
33	**Anatoliaca3-10**	0.012	0.045	0.039	0.042	0.036	0.036		1 / 0 / 0	1 / 0 / 0	8 / 0 / 3	8 / 0 / 2	9 / 0 / 2	7 / 0 / 2
34	**Anatoliaca3-11**	0.009	0.042	0.036	0.039	0.033	0.033	0.003		2 / 0 / 0	7 / 0 / 3	7 / 0 / 2	8 / 0 / 2	6 / 0 / 2
35	**Anatoliaca3-12**	0.015	0.049	0.042	0.045	0.039	0.039	0.003	0.006		9 / 0 / 3	9 / 0 / 2	10 / 0 / 2	8 / 0 / 2
36	**Cerigensis1-11**	0.018	0.042	0.042	0.039	0.033	0.033	0.024	0.021	0.027		2 / 0 / 1	3 / 0 / 1	1 / 0 / 1
37	**Cerigensis1-12**	0.018	0.045	0.045	0.042	0.036	0.036	0.024	0.021	0.027	0.006		1 / 0 / 0	1 / 0 / 0
38	**Cerigensis1-21**	0.021	0.049	0.049	0.045	0.039	0.039	0.027	0.024	0.030	0.009	0.003		2 / 0 / 0
39	**Cerigensis1-10**	0.015	0.042	0.042	0.039	0.033	0.033	0.021	0.018	0.024	0.003	0.003	0.006	
40	**Trodos Cyprus**	0.064	0.074	0.081	0.077	0.071	0.071	0.058	0.061	0.055	0.064	0.064	0.068	0.061
41	**Rana cretensis**	0.131	0.153	0.146	0.149	0.153	0.156	0.131	0.135	0.128	0.139	0.139	0.142	0.135
42	**Rana epeirotica**	0.156	0.160	0.153	0.156	0.153	0.153	0.142	0.146	0.139	0.156	0.156	0.160	0.153
43	**Rana lessonae**	0.156	0.175	0.175	0.171	0.167	0.167	0.164	0.160	0.160	0.164	0.156	0.160	0.160
44	**Syria Abu Kemal 313**	0.024	0.045	0.045	0.042	0.042	0.042	0.024	0.021	0.027	0.030	0.030	0.033	0.027
45	**Rana bedriagae 320**	0.039	0.055	0.055	0.052	0.052	0.052	0.052	0.049	0.049	0.039	0.039	0.042	0.036
46	**Rana bedriagae 322**	0.039	0.055	0.055	0.052	0.052	0.052	0.045	0.042	0.042	0.039	0.039	0.042	0.036
47	**Syria Abu Kemal 312**	0.021	0.042	0.042	0.039	0.039	0.039	0.021	0.018	0.024	0.027	0.027	0.030	0.024

Table A continued.

		40	41	42	43	44	45	46	47
1	**Caralitana1-10**	17 / 5 / 4	39 / 8 / 10	37 / 9 / 7	42 / 8 / 5	8 / 5 / 4	11 / 5 / 2	11 / 5 / 3	7 / 5 / 4
2	**Caralitana3-11**	18 / 4 / 5	40 / 7 / 11	39 / 8 / 8	42 / 7 / 4	9 / 4 / 5	12 / 4 / 3	12 / 4 / 4	8 / 4 / 5
3	**Caralitana3-10**	17 / 4 / 4	39 / 7 / 10	38 / 8 / 7	43 / 7 / 5	8 / 4 / 4	11 / 4 / 2	11 / 4 / 3	7 / 4 / 4
4	**Anatoliaca2-10**	17 / 1 / 2	38 / 4 / 8	38 / 7 / 7	43 / 4 / 3	8 / 1 / 2	13 / 1 / 0	13 / 1 / 1	7 / 1 / 2
5	**Caralitana4-10**	18 / 4 / 5	38 / 7 / 9	37 / 8 / 6	44 / 7 / 6	7 / 4 / 3	12 / 4 / 3	12 / 4 / 4	6 / 4 / 3
6	**Caralitana1-11**	17 / 5 / 4	39 / 8 / 10	37 / 9 / 7	42 / 8 / 5	8 / 5 / 4	11 / 5 / 2	11 / 5 / 3	7 / 5 / 4
7	**Caralitana4-11**	19 / 4 / 5	39 / 7 / 9	38 / 8 / 6	45 / 7 / 6	8 / 4 / 3	13 / 4 / 3	13 / 4 / 4	7 / 4 / 3
8	**Ceyhanensis1-10**	16 / 1 / 3	34 / 4 / 9	40 / 7 / 8	41 / 4 / 4	14 / 3 / 3	13 / 1 / 1	13 / 1 / 2	13 / 3 / 3
9	**Ceyhanensis1-21**	16 / 1 / 3	34 / 4 / 9	40 / 7 / 8	41 / 4 / 4	14 / 3 / 3	13 / 1 / 1	13 / 1 / 2	13 / 3 / 3
10	**Ceyhanensis1-31**	17 / 1 / 3	35 / 4 / 9	39 / 7 / 8	42 / 4 / 4	13 / 3 / 3	14 / 1 / 1	14 / 1 / 2	14 / 3 / 3
11	**Ceyhanensis1-32**	15 / 1 / 3	31 / 4 / 9	38 / 7 / 8	42 / 4 / 4	15 / 3 / 3	12 / 1 / 1	12 / 1 / 2	14 / 3 / 3
12	**Ceyhanensis1-11**	17 / 1 / 3	33 / 4 / 9	39 / 7 / 8	42 / 4 / 4	15 / 3 / 3	14 / 1 / 1	14 / 1 / 2	14 / 3 / 3
13	**Caralitana1-12**	17 / 5 / 4	39 / 8 / 10	37 / 9 / 7	42 / 8 / 5	8 / 5 / 4	11 / 5 / 2	11 / 5 / 3	7 / 5 / 4
14	**Caralitana1-13**	18 / 5 / 5	40 / 8 / 11	38 / 9 / 8	43 / 8 / 6	9 / 5 / 5	12 / 5 / 3	12 / 5 / 4	8 / 5 / 5
15	**Caralitana1-14**	16 / 5 / 4	38 / 8 / 10	36 / 9 / 7	41 / 8 / 5	9 / 5 / 4	10 / 5 / 2	10 / 5 / 3	8 / 5 / 4
16	**Caralitana1-21**	19 / 5 / 4	41 / 8 / 10	37 / 9 / 7	40 / 8 / 5	10 / 5 / 4	13 / 5 / 2	13 / 5 / 3	9 / 5 / 4
17	**Caralitana1-15**	16 / 5 / 4	38 / 8 / 10	36 / 9 / 7	41 / 8 / 5	9 / 5 / 4	12 / 5 / 2	12 / 5 / 3	8 / 5 / 4
18	**Caralitana1-16**	18 / 5 / 5	40 / 8 / 10	38 / 9 / 8	43 / 8 / 5	9 / 5 / 5	12 / 5 / 3	12 / 5 / 4	8 / 5 / 5
19	**Caralitana3-12**	18 / 5 / 5	38 / 8 / 9	36 / 9 / 6	43 / 8 / 6	7 / 5 / 3	12 / 5 / 3	12 / 5 / 4	6 / 5 / 3
20	**Anatoliaca2-11**	18 / 1 / 2	39 / 9 / 8	39 / 7 / 7	42 / 4 / 3	9 / 1 / 2	14 / 1 / 0	14 / 1 / 1	8 / 1 / 2
21	**Caralitana5-10**	20 / 4 / 6	37 / 7 / 10	37 / 8 / 7	43 / 7 / 7	11 / 4 / 4	16 / 4 / 4	17 / 4 / 5	10 / 4 / 4
22	**Anatoliaca1-10**	20 / 1 / 3	39 / 4 / 8	41 / 7 / 8	46 / 4 / 4	7 / 1 / 3	14 / 1 / 1	14 / 1 / 2	6 / 1 / 3
23	**Anatoliaca1-11**	19 / 1 / 2	38 / 4 / 8	40 / 7 / 7	45 / 4 / 3	6 / 1 / 2	13 / 1 / 0	13 / 1 / 1	5 / 1 / 2

Table A continued.

24	**Ridibunda1-10**	28 / 2 / 3	36 / 5 / 9	38 / 6 / 6	47 / 5 / 4	20 / 4 / 3	18 / 2 / 1	18 / 2 / 2	22 / 4 / 3
25	**Anatoliaca1-21**	18 / 1 / 2	39 / 4 / 8	41 / 7 / 7	46 / 4 / 3	7 / 1 / 2	14 / 1 / 0	14 / 1 / 1	4 / 1 / 2
26	**Anatoliaca1-22**	18 / 1 / 2	37 / 4 / 8	41 / 7 / 7	44 / 4 / 3	7 / 1 / 2	12 / 1 / 0	12 / 1 / 1	6 / 1 / 2
27	**Anatoliaca1-23**	20 / 1 / 2	37 / 4 / 8	41 / 7 / 7	44 / 4 / 3	7 / 1 / 2	12 / 1 / 0	12 / 1 / 1	6 / 1 / 2
28	**Caralitana2-10**	19 / 5 / 6	39 / 8 / 10	40 / 9 / 7	45 / 8 / 7	10 / 5 / 4	13 / 5 / 4	13 / 5 / 5	9 / 5 / 4
29	**Caralitana2-21**	21 / 5 / 6	37 / 8 / 10	38 / 9 / 7	45 / 8 / 7	10 / 5 / 4	13 / 5 / 4	13 / 5 / 5	9 / 5 / 4
30	**Caralitana2-11**	20 / 5 / 6	38 / 8 / 10	39 / 9 / 7	44 / 8 / 7	9 / 5 / 4	12 / 5 / 4	12 / 5 / 5	8 / 5 / 4
31	**Caralitana1-17**	17 / 6 / 4	38 / 9 / 10	37 / 10 / 7	42 / 9 / 5	8 / 6 / 4	11 / 6 / 2	11 / 6 / 3	7 / 6 / 4
32	**Caralitana1-18**	18 / 5 / 5	40 / 8 / 11	38 / 9 / 8	43 / 8 / 6	9 / 5 / 5	12 / 5 / 3	12 / 5 / 4	8 / 5 / 5
33	**Anatoliaca3-10**	18 / 1 / 3	37 / 4 / 9	37 / 7 / 6	46 / 4 / 4	7 / 1 / 1	16 / 1 / 1	14 / 1 / 0	6 / 1 / 1
34	**Anatoliaca3-11**	19 / 1 / 3	38 / 4 / 9	38 / 7 / 6	45 / 4 / 4	6 / 1 / 1	15 / 1 / 1	13 / 1 / 0	5 / 1 / 1
35	**Anatoliaca3-12**	17 / 1 / 3	36 / 4 / 9	36 / 7 / 6	45 / 4 / 4	8 / 1 / 1	15 / 1 / 1	13 / 1 / 0	7 / 1 / 1
36	**Cerigensis1-11**	20 / 1 / 4	39 / 4 / 10	41 / 7 / 9	46 / 4 / 5	9 / 1 / 4	12 / 1 / 2	12 / 1 / 3	8 / 1 / 4
37	**Cerigensis1-12**	20 / 1 / 3	39 / 4 / 9	41 / 7 / 8	44 / 4 / 4	9 / 1 / 3	12 / 1 / 1	12 / 1 / 2	8 / 1 / 3
38	**Cerigensis1-21**	21 / 1 / 3	40 / 4 / 9	42 / 7 / 8	45 / 4 / 4	10 / 1 / 3	13 / 1 / 1	13 / 1 / 2	9 / 1 / 3
39	**Cerigensis1-10**	19 / 1 / 3	38 / 4 / 9	40 / 7 / 8	45 / 4 / 4	8 / 1 / 3	11 / 1 / 1	11 / 1 / 2	7 / 1 / 3
40	**Trodos Cyprus**		36 / 3 / 6	42 / 6 / 9	42 / 3 / 5	23 / 2 / 4	18 / 0 / 2	18 / 0 / 3	20 / 2 / 4
41	**Rana cretensis**	0.124		44 / 9 / 13	54 / 4 / 10	42 / 5 / 8	38 / 3 / 8	38 / 3 / 9	41 / 5 / 8
42	**Rana epeirotica**	0.156	0.175		45 / 7 / 8	41 / 8 / 5	39 / 6 / 7	38 / 6 / 6	40 / 8 / 5
43	**Rana lessonae**	0.146	0.194	0.171		47 / 5 / 5	41 / 3 / 3	42 / 3 / 4	46 / 5 / 5
44	**Syria Abu Kemal 313**	0.077	0.153	0.160	0.171		17 / 2 / 2	15 / 2 / 1	3 / 0 / 0
45	**Rana bedriagae 320**	0.055	0.131	0.146	0.142	0.058		2 / 0 / 1	16 / 2 / 2
46	**Rana bedriagae 322**	0.055	0.131	0.142	0.146	0.052	0.006		14 / 2 / 1
47	**Syria Abu Kemal 312**	0.068	0.149	0.156	0.167	0.009	0.055	0.049	

APPENDIX B: Table of Sampling Information

Table B. Giving information about detail of sampling process : Sample ID, Haplotype ID, names of their source population and their location.

Sample ID	Haplotype ID	Population	City
CBCAST421	A1-11	Seydişehir	Konya
CBCAST072	C1-10	Antalya	Antalya
CBCAST074	C1-10	Antalya	Antalya
CBCAST075	C3-11	Antalya	Antalya
CBCAST076	C1-10	Antalya	Antalya
CBCAST327	C1-10	Karacaörenler	Isparta
CBCAST328	C1-10	Karacaörenler	Isparta
CBCAST329	C1-10	Karacaörenler	Isparta
CBCAST3210	C1-10	Karacaörenler	Isparta
CBCAST3211	C1-10	Karacaörenler	Isparta
CBCAST3212	C1-10	Karacaörenler	Isparta
CBCAST3213	C1-10	Karacaörenler	Isparta
CBCAST3214	C1-10	Kovada	Isparta
CBCAST3215	A1-11	Kovada	Isparta
CBCAST3216	C3-10	Kovada	Isparta
CBCAST1517	C1-10	Burdur Lake	Burdur
CBCAST1519	C1-10	Burdur Lake	Burdur
CBCAST1520	C1-10	Burdur Lake	Burdur
CBCAST2021	A2-10	Gemiş	Denizli
CBCAST2022	A2-10	Gemiş	Denizli
CBCAST2023	A2-10	Gemiş	Denizli
CBCAST2024	A2-10	Gemiş	Denizli
CBCAST2025	C1-10	Gemiş	Denizli
CBCAST2026	A2-10	Gemiş	Denizli
CBCAST2027	A2-10	Gemiş	Denizli
CBCAST2028	A2-10	Gemiş	Denizli
CBCAST2029	A2-10	Gemiş	Denizli

Table B continued.

CBCAST2031	A2-10	Gemiş	Denizli
CBCAST3233	A1-11	Eğirdir Lake	Isparta
CBCAST3234	C1-10	Eğirdir Lake	Isparta
CBCAST3238	C3-10	Eğirdir Lake	Isparta
CBCAST3239	C3-10	Eğirdir Lake	Isparta
CBCAST4240	C4-10	Akşehir-Eber Lakes	Afyon/Konya
CBCA3241	C1-10	Kovada	Isparta
CBCA3242	C1-11	Kovada	Isparta
CBCA0743	C1-10	Antalya	Antalya
CBCA6444	A2-10	Sorgun Dam	Uşak
CBCA6445	A2-10	Sorgun Dam	Uşak
CBCA6446	A2-10	Sorgun Dam	Uşak
CBCA2047	C4-10	Işıklı Lake	Denizli
CBCA2048	C3-10	Işıklı Lake	Denizli
CBCA2049	C4-11	Işıklı Lake	Denizli
CBCA2050	C4-10	Işıklı Lake	Denizli
CBCA2051	C3-10	Işıklı Lake	Denizli
CBCA2052	C4-10	Işıklı Lake	Denizli
TBCASE0154	CEY1-10	Yumurtalık	Adana
TBCASE0155	CEY1-10	Yumurtalık	Adana
TBCASE0156	CEY1-21	Yumurtalık	Adana
TBCASE0157	CEY1-31	Yumurtalık	Adana
TBCASE0158	CEY1-31	Yumurtalık	Adana
TBCASE0159	CEY1-10	Yumurtalık	Adana
TBCASE0160	CEY1-32	Yumurtalık	Adana
TBCASE0161	CEY1-21	Yumurtalık	Adana
TBCASE0162	CEY1-21	Yumurtalık	Adana
TBCASE0164	CEY1-11	Yumurtalık	Adana
TBCASE0166	CEY1-10	Yumurtalık	Adana
MTAECA1568	C1-10	Bucak	Burdur
MTAECA1569	C1-10	Bucak	Burdur
MTAECA1570	C1-10	Bucak	Burdur
MTAECA1571	C1-10	Bucak	Burdur
MTAECA1572	C3-10	Bucak	Burdur
MTAECA1573	C1-10	Bucak	Burdur

Table B continued.

MTAECA1574	C4-10	Bucak	Burdur
MTAECA1575	C3-10	Bucak	Burdur
MTAECA1576	C1-10	Bucak	Burdur
MTAECA1577	C1-10	Bucak	Burdur
ISCA1578	A2-10	Yeşilova	Burdur
ISCA1579	C4-10	Yeşilova	Burdur
ISCA1580	C1-10	Yeşilova	Burdur
ISCA1581	C1-10	Yeşilova	Burdur
ISCA1582	A2-10	Yeşilova	Burdur
ISCA1583	A2-10	Yeşilova	Burdur
ISCA1584	A2-10	Yeşilova	Burdur
ISCA1585	A2-10	Yeşilova	Burdur
ISCA1586	A2-10	Yeşilova	Burdur
ISCA1587	A2-10	Yeşilova	Burdur
ISCA1588	A2-10	Yeşilova	Burdur
OAOBBKCA1593	C1-10	Bucak	Burdur
OAOBBKCA1594	C1-10	Bucak	Burdur
OAOBBKCA1596	C1-10	Bucak	Burdur
OBBKCA1597	C1-10	Bucak	Burdur
OBBKCA1598	C1-12	Bucak	Burdur
OBBKCA1599	C1-10	Bucak	Burdur
OBBKCA15101	C1-10	Bucak	Burdur
OBBKCA15102	C1-10	Bucak	Burdur
OBBKCA32104	C1-10	Kovada	Isparta
OBBKCA32105	C1-10	Kovada	Isparta
OBBKCA32106	C1-10	Kovada	Isparta
OBBKCA32107	C1-10	Kovada	Isparta
OBBKCA32108	C1-10	Kovada	Isparta
HKHSBKCA07111	C1-13	Antalya	Antalya
HKHSBKCA07112	C1-10	Antalya	Antalya
HKHSBKCA07113	C1-10	Antalya	Antalya
HKHSBKCA07114	C1-10	Antalya	Antalya
HKHSBKCA07115	C1-10	Antalya	Antalya
HKHSBKCA07116	C1-10	Antalya	Antalya
HKHSBKCA07118	C1-14	Antalya	Antalya

Table B continued.

HKHSBKCA07119	C1-10	Antalya	Antalya
HKHSBKCA07120	C1-21	Antalya	Antalya
HKHSBKCA07121	C1-15	Antalya	Antalya
HKHSBKCA07122	C1-10	Antalya	Antalya
HKHSBKCA07123	C1-13	Antalya	Antalya
HKHSBKCA07124	C1-10	Antalya	Antalya
VDBKCA07125	C1-10	Korkuteli	Antalya
VDBKCA07126	A2-10	Korkuteli	Antalya
VDBKCA07127	C1-10	Korkuteli	Antalya
VDBKCA07128	C1-16	Korkuteli	Antalya
VDBKCA07129	C4-10	Korkuteli	Antalya
VDBKCA07130	C4-10	Korkuteli	Antalya
VDBKCA07131	C1-10	Korkuteli	Antalya
VD07134	C1-10	Gözlemevi	Antalya
VD07135	C1-10	Gözlemevi	Antalya
VD07136	C1-10	Gözlemevi	Antalya
MHSACA15137	A2-10	Kemer	Burdur
MHSACA15138	C1-10	Kemer	Burdur
MHSACA15139	C1-16	Kemer	Burdur
MHSACA15140	C1-10	Kemer	Burdur
MHSACA15141	C1-10	Kemer	Burdur
MHSACA15142	C1-10	Kemer	Burdur
MHSACA15143	C1-10	Kemer	Burdur
YECA15144	C5-10	Tefenni	Burdur
YECA15145	C4-10	Tefenni	Burdur
YECA15146	A2-10	Tefenni	Burdur
KYECA15147	A2-10	Tefenni	Burdur
KYECA15148	A2-10	Tefenni	Burdur
KYECA15149	C4-10	Tefenni	Burdur
KYECA15150	C3-12	Tefenni	Burdur
MAHACA15151	A2-11	Tefenni	Burdur
MAHACA15152	A2-10	Tefenni	Burdur
CCYZCA20153	A2-10	Gökpınar Dam	Denizli
CCYZCA20155	A2-10	Gökpınar Dam	Denizli
CCYZCA20156	A2-10	Gökpınar Dam	Denizli

Table B continued.

CCYZCA20157	A2-10	Gökpınar Dam	Denizli
HS07165	CER1-12	Avlan Lake	Antalya
HS07166	CER1-12	Avlan Lake	Antalya
HS07167	C5-10	Avlan Lake	Antalya
HS07168	C5-10	Avlan Lake	Antalya
HS07169	C5-10	Avlan Lake	Antalya
TBOECA68170	A1-10	Eşmekaya	Aksaray
TBOECA68171	A1-10	Eşmekaya	Aksaray
TBOECA68172	A1-10	Eşmekaya	Aksaray
TBOECA68173	A1-10	Eşmekaya	Aksaray
OECA68174	A1-11	Eşmekaya	Aksaray
OECA68175	C4-10	Eşmekaya	Aksaray
OECA68176	C4-10	Eşmekaya	Aksaray
MTHTCA07177	C1-10	Alanya	Antalya
MTHTCA07178	C1-10	Alanya	Antalya
MACA22179	RD1-10	Edirne	Edirne
MACA22180	RD1-10	Edirne	Edirne
MACA22181	RD1-10	Edirne	Edirne
MACA22182	RD1-10	Edirne	Edirne
MACA22183	A1-21	Edirne	Edirne
CBCA03184	A1-11	Akşehir-Eber Lakes	Afyon/Konya
CBCA03185	A2-10	Akşehir-Eber Lakes	Afyon/Konya
CBCA03186	A1-11	Akşehir-Eber Lakes	Afyon/Konya
CBCA03187	C4-10	Akşehir-Eber Lakes	Afyon/Konya
CBCA03188	A1-22	Akşehir-Eber Lakes	Afyon/Konya
CBCA03189	A2-10	Akşehir-Eber Lakes	Afyon/Konya
CBCA03190	C4-10	Akşehir-Eber Lakes	Afyon/Konya
ATDC08191	A1-23	Artvin	Artvin
ATDC08192	A1-23	Artvin	Artvin
ATDC08193	A1-23	Artvin	Artvin
ATDC08194	A1-23	Artvin	Artvin
MEFUCA42195	C2-21	Beyşehir	Konya
MEFUCA42196	C2-11	Beyşehir	Konya
MEFUCA42197	C2-11	Beyşehir	Konya
MEFUCA42198	C2-11	Beyşehir	Konya

Table B continued.

SGCA42199	A1-11	Seydişehir	Konya
SGCA42201	C2-11	Seydişehir	Konya
SGCA42202	C4-10	Seydişehir	Konya
OKCA42203	C2-11	İvriz	Konya
OYCA70204	C1-10	Gödet Dam	Karaman
OYCA70206	C1-17	Gödet Dam	Karaman
OYCA70207	C1-10	Gödet Dam	Karaman
OYCA70208	C1-18	Gödet Dam	Karaman
AKCA48209	A3-10	Marmaris	Muğla
AKCA48210	A3-11	Marmaris	Muğla
AKCA48211	A3-11	Marmaris	Muğla
AKCA48212	A3-12	Marmaris	Muğla
AKCA48213	CER1-11	Fethiye	Muğla
AKCA48214	CER1-12	Fethiye	Muğla
AKCA48215	CER1-21	Fethiye	Muğla
AKCA48216	CER1-11	Fethiye	Muğla
CA07217	CER1-10	Kaş	Antalya
CA07218	CER1-12	Kaş	Antalya
CA07219	CER1-12	Kaş	Antalya
OK42220	C2-11	İvriz	Konya
OK42221	C2-11	İvriz	Konya
OK42222	C2-11	İvriz	Konya
SOCA33224	CEY1-21	Mersin	İçel
SOCA33225	CEY1-21	Mersin	İçel
SOCA33226	CEY1-21	Mersin	İçel
SOCA33227	CEY1-21	Mersin	İçel
KKOBCA06229	A1-10	Ankara	Ankara
KKOBCA06230	A1-10	Ankara	Ankara