DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

A PRELIMINARY INVESTIGATION ON GENETICS OF SMALL CETACEANS IN TURKEY

by

Reyhan SÖNMEZ

August, 2011

İZMİR

A PRELIMINARY INVESTIGATION ON GENETICS OF SMALL CETACEANS IN TURKEY

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by

Reyhan SÖNMEZ

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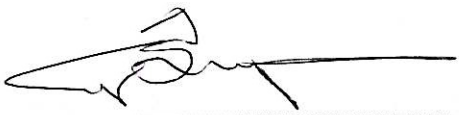
İZMİR

M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"A PRELIMINARY INVESTIGATION ON GENETICS OF SMALL CETACEANS IN TURKEY"** completed by **REYHAN SÖNMEZ** under supervision of **ASSIST. PROF. DR. KEMAL CAN BİZSEL** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.



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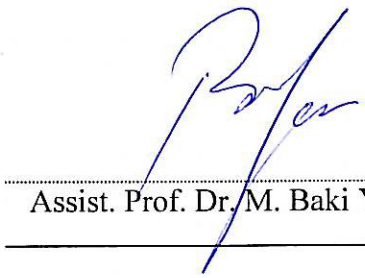

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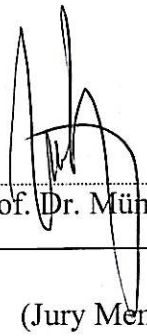

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A PRELIMINARY INVESTIGATION ON GENETICS OF SMALL CETACEANS IN TURKEY

ABSTRACT

In this study Black Sea harbour porpoise's population genetic structure was investigated based on a new genetic study carried out and incorporating published records. Also collected bottlenose dolphin and striped dolphin samples' mitochondrial DNA nucleotide sequences were compared with the published sequences.

The present study is aimed at examining the phylogenetic structure of the Black Sea harbour porpoise population, focusing on the variations of nucleotide sequences in the mitochondrial DNA, namely 16S rDNA, COI and Dloop. Phylogenetic structure was determined using maximum parsimony, maximum likelihood and bayesian inference methods.

The results show that the Black Sea and the Aegean Sea samples genetically differed from other populations of the same species in different oceans. Consensus trees given by both maximum parsimony and maximum likelihood for Dloop sequences showed a distinct group formation. This genetically distinct group had samples from West Black Sea coasts of Turkey including sampling areas Karaburun, Rumeli Feneri and İğneada. But because not all of the samples from the same location were found together in certain phylogenetic groups, it was not possible talk about distinct subpopulation formations. COI results revealed that the Black Sea samples were not significantly different from each other.

Keywords*:* Phocoena phocoena, mtDNA, phylogenetic structure

TÜRKİYE SULARINDAKİ KÜÇÜK DENİZ MEMELİLERİNİN GENETİĞİ ÜZERİNE BİR ÖN ÇALIŞMA

ÖZ

Tez çalışması kapsamında Karadeniz muturlarının popülasyon genetik yapısı, yapılan yeni bir genetik çalışma ve literatür kayıtlarına dayanarak araştırılmıştır. Ayrıca şişeburunlu ve çizgili yunus örneklerinin mitokondriyal DNA nükleotid dizileri literatürdeki kayıtlar ile karşılaştırılmıştır.

Çalışmada mitokondriyal DNA nükleotid dizilerinin 16S rDNA, COI ve Dloop bölgelerindeki varyasyonlar incelenerek, Karadeniz mutur popülasyonunun filogenetik yapısı ortaya konmaya çalışılmıştır. Filogenetik yapı maksimum parsimoni, maksimum benzerlik ve bayesian çıkarım metodları kullanılarak incelenmiştir.

Çalışmanın sonuçlarına göre Karadeniz ve Ege örnekleri, aynı türün diğer okyanuslarda yaşayan popülasyonlarından genetik olarak farklılaşmıştır . Dloop için yapılan maksimum parsimoni ve maksimum benzerlik analizlerine göre, Türkiye'nin Batı Karadeniz bölgesinde, Karaburun, Rumeli Feneri ve İğneada kıyılarından bazı örnekleri kapsayan bir grubun diğer örneklerden genetik olarak farklılaştığı bulunmuştur. Ancak aynı bölgeye ait örneklerin hepsi aynı şekilde, tek bir filogenetik grup içerisinde toplanmadığından farklı altpopulasyon yapılarından söz etmek mümkün olmamıştır. COI gen bölgesi sonuçlarına göre Karadeniz içinde anlamlı bir farklılaşma gözlemlenmemiştir.

Anahtar Sözcükler*:* Phocoena phocoena, mtDNA, filogenetik yapı

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CHAPTER ONE
INTRODUCTION

The common bottlenose dolphin (*Tursiops truncatus,* (Montagu, 1821)), short beaked common dolphin (*Delphinus delphis,* Linnaeus, 1758) and harbour porpoise (*Phocoena phocoena,* (Linnaeus, 1758)) are three small cetacean species which have some distinct population structures in Turkish Waters. Based on morphological and/or genetic studies there are three subspecies of each species, mainly inhabiting the Black Sea. These subspecies are the Black Sea bottlenose dolphin (*Tursiops truncatus ponticus,* (Barabash-Nikiforov, 1940)), the Black Sea short beaked common dolphin (*Delphinus delphis ponticus,* (Barabash-Nikiforov, 1935)) and the Black Sea harbour porpoise (*Phocoena phocoena relicta,* (Abel, 1905)) (Notarbartolo di Sciara & Birkun, 2010). Additional information about these species can be found in Annex A.

The conservation status of cetaceans in the Black and Mediterranean Seas has been a challenging issue for many years, although they are protected by environmental laws, multinational agreements and international conservation organizations such as IUCN (International Union for the conservation of nature), ACCOBAMS (Agreement on the conservation of cetaceans of the Black Sea, Mediterranean Sea and contiguous Atlantic area) and IWC (International Whaling Commission). These three small cetacean species have been listed in the IUCN Red List of Threatened Species for several decades. The Black Sea subspecies of the harbour porpoise (*Phocoena phocoena relicta*) and the Black Sea subspecies of the common bottlenose dolphin (*Tursiops truncatus ponticus*) have been both classified as endangered since 2008. The Mediterranean subpopulation of short beaked common dolphin (*Delphinus delphis*) has been classified as endangered since 2003 while the Black Sea subpopulation of the short beaked common dolphin (*Delphinus delphis ponticus*) has been classified as vulnerable since 2008.

The genetic structure of the Black Sea harbour porpoises was investigated in the Black Sea by means of a new genetic study which also used published records. Also a sample from the Aegean coasts of Turkey was analysed with the Black Sea samples. Collected bottlenose and striped dolphin samples were not subjected into phylogenetic analysis, but nucleotide sequences were compared with the published sequences from different sampling areas.

There has been an increase of terms used to describe groups of individuals below the species level. This imprecise terminology leads to misunderstanding and disagreement among parts of the scientific community (Tansley, 1935; Whittaker *et al.*, 1975). To avoid the misunderstanding the concepts of population, subspecies, subpopulation, management units and evolutionary significant units, used in this study are defined in Annex B in detail.

mtDNA of higher animals meet the criteria of desired properties for an ideal molecular system for population genetics. It is distinctive and ubiquitously distributed, easy to isolate, maternally inherited, as well as having a simple genetic structure and ability to evolve rapidly. Beside its practical advantages for laboratory work, these characteristics enable homologous comparisons among organisms and exploration of new character states arising within the lifespan of a species. Thus, mtDNA is a widely used marker in population genetic studies (Avise *et al.*, 1987).

The different genes within the mitochondrial genome evolve at different rates and therefore different genes can be used in specific analyses. The more slowly evolving genes are often used for phylogenetic analysis while the more rapidly evolving regions tend to be used for population studies (Avise *et al.*, 1987; Aquadro & Greenberg, 1983; Baker *et al.*, 1993; Brown *et al.*, 1979; Stevens *et al.*, 1989). Previous studies of mtDNA have shown that populations are often partitioned into phylogeographic units based on geographic distance, the presence of topographical boundaries between populations or behavioural differences (Avise *et al.*, 1987).

Sequencing of mtDNA Dloop (Control region) has proven useful in population genetics and understanding evolutionary relationships in a variety of marine mammal species (Baker *et al.,* 1993) given its rapid evolving rate which is 5 to 10 times higher than single-copy nuclear DNA. Similarly 16S rDNA sequence data has been used to investigate population structures and phylogeny (Amann *et al.,* 1995). The mitochondrial cytochrome c oxidase subunit (COI) sequence, which appears to be among the most conservative protein coding genes in the mitochondrial genome of animals, was used for phylogenetic analysis. Hebert *et al.,* (2003), have suggested that a DNA-based identification system, founded on the mitochondrial gene, COI, can help resolve the taxonomic status of species; sequence divergences of COI regularly enable the discrimination of closely allied species in all animal phyla.

The present study was aimed at examining the phylogenetic structure of the Black Sea harbour porpoise population, focusing on the variations of nucleotide sequences in the mitochondrial DNA (mtDNA), namely Dloop, COI and 16S rDNA.

Phylogenetic analysis is the investigation of the evolution and relationships among organisms that is widely used in comparative genomics (Salemi & Vandamme, 2003). In molecular based phylogenetic analysis, the relationship between samples is estimated by inferring the common history of their genes and then phylogenetic trees are constructed to illustrate evolutionary relationships among genes and organisms (Kidd & Zonta, 1971).

There are various phylogenetic tree construction and phylogenetic analysis methods using different strategies. In general, there are three basic methods that have been used to estimate phylogeny, which are distance, maximum parsimony (MP), and maximum likelihood (ML). The relative merits of these methods have been discussed for a number of years (Faith, 1985; Kunhner & Felsenstein, 1994; Huelsenbeck, 1995; Farris *et al.,* 1996; Lewis, 1998; Steel & Penny, 2000).

Every method has its own advantages, disadvantages and outperforms in comparison with other methods. In this study, we used the maximum parsimony (MP), maximum likelihood (ML) and bayesian inference (BI) to construct phylogenetic trees. Each of the methods, "out performs" the others. For instance, it is now generally accepted that when rates of change long branches vary greatly, employing a parsimony optimality criterion may be misleading due to "long branch attraction" (Felsenstein, 1985; Siddall, 1998) whereas additional studies have shown that ML may be inconsistent in other situations, such as when the chosen model of evolution is inappropriate (e.g., Farris, 1999). Under most sets of realistic conditions, comparison of ML and MP indicates that these methods perform similarly and often result in highly concordant topologies (Kimball *et al*., 2003). Bayesian analyses which was proposed recently in 1996, is now receiving much attention in the literature (Huelsenbeck & Ronquist, 2001; Huelsenbeck *et al.*, 2002; Lewis, 2001). Bayesian inference differs from other methods of phylogenetic inference with major differences between bayesian and classical statistics. Classical statistics use current data to test specific hypotheses while bayesian statistics differ in that in addition to the current data, prior knowledge is included in the testing of the hypothesis. The prior probability distribution of trees and can be viewed as either a positive or negative attribute depending upon the strength and legitimacy of the prior expectation (Archibald *et al.*, 2003).

The study aimed to reveal potential management units within the study area. According to Birkun (2002), the species diversity of Black Sea fauna is found to be lower than the Mediterranean Sea. Specific features of the Black Sea make it very vulnerable to disturbances of its environment and ecosystems. Eutrophication, pollution, and irresponsible fishing are the main factors resulted in an overall decline of biological resources and the diversity of species. The top predator populations in such threatened ecosystem should be monitored. Gathered genetic data can aid to identify the potential management units as the genetic distinctness of a population, has long been recognized as a key to conservation concerns (Moritz, 1995).

CHAPTER TWO
MATERIAL AND METHODS

2.1 Materials

Samples used in this study were collected along the Black Sea coasts of Turkey from harbour porpoise individuals incidentally taken in turbot fisheries and found stranded on beaches between March 2010 and May 2011. Three *Tursiops truncatus*, two *Stenella coerualba* and one *Delphinus delphis* sample which were previously collected for future genetic analysis were added to this study as they were in the geographical scope of the study. The total number of individuals analysed were 55. Figure 2.1 represents the distribution of the 49 harbour porpoise, 3 bottlenose dolphins, 2 striped dolphins and 1 common dolphin which were included to the study. Table 2.1 represents samples' geographical location.

Table 2.1 Geographical locations of collected samples; P.p refers to *Phocoena phocoena*, T.t refers to *Tursiops truncatus*, D.d refers to *Delphinus delphis*, S.c refers to *Stenella coeruleoalba*

Sample Number	Location	Species	Date of Sampling
1	Romania	P.p	March 2010
2	Ereğli / Zonguldak	P.p	April 2010
3	Şile / İstanbul	P.p	April 2010
4	Ereğli / Zonguldak	P.p	April 2010
5	Lapseki / Çanakkale	T.t	August 2009
6	Kuşadası / Aydın	T.t	March 2009
7	Sinop	P.p	April 2010
8	İğneada / Kırklareli	P.p	July 2009
9	Urla / İzmir	P.p	October 2006
10	Urla / İzmir	S.c	February 2009
11	Kartal / İstanbul	D.d	April 2010
12-13	Zonguldak	P.p	April 2010
14	Karaburun / İstanbul	P.p	May 2010
15-16	Russia	P.p	May 2010
17	Kefken / Kocaeli	P.p	May 2010
18	Karaburun / İstanbul	P.p	May 2010
19	Kıyıköy / Kırklareli	P.p	May 2010
20-22	Kıyıköy / Kırklareli	P.p	May 2010
23	Kuşadası / İzmir	S.c	June 2010
24	Fatsa / Ordu	P.p	April 2010
25	Fatsa / Ordu	P.p	May 2010
26	Fatsa / Ordu	T.t	April 2010
27-34	Karaburun / İstanbul	P.p	June 2010
35-49	Karaburun / İstanbul	P.p	May 2011
50-51	Rumeli feneri / İstanbul	P.p	May 2011
52-55	Şile / İstanbul	P.p	May 2011

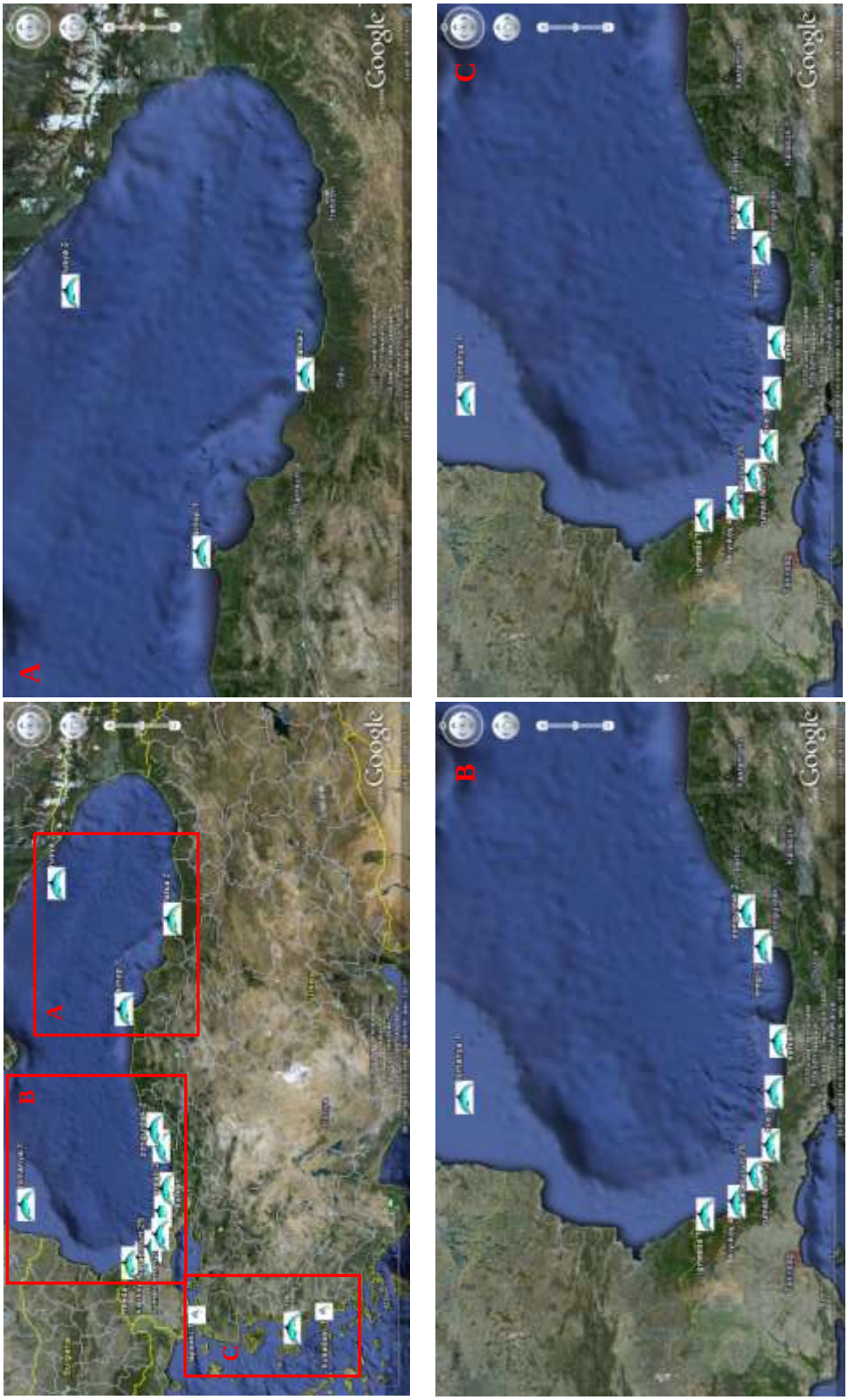

Figure 2.1 Geographical distribution of samples

Tissue samples from stranded animals were collected by local fishermen or by myself whenever it was possible. Tissue samples of bycaught animals were collected by local fishermen also and preserved at -20°C until shipping in styrofoam boxes with ice cubes around the sample. As soon as the parcel arrived at the laboratory, skin and muscle tissue samples were taken from the flesh and preserved in absolute ethanol at +4 °C until DNA extraction. Chemical solutions used in this study listed in Table 2.2.

Table 2.2 Chemical solutions used in this study

#	Solution	Preperation
	Manuel DNA Isolation	
1	Protainase K	10 mg/ml Protainase K in sterilized distilled water
2	SDS-Lysis Buffer (pH 8,0)	50 mM Tris (pH 8.0) 50 mM Sucrose, 100 mM NaCl, 50 mM Na_2 EDTA (pH 7,4) 50 mM Tris (pH 8.0) 50 mM Sucrose, 100 mM NaCl, 50 mM Na_2 EDTA (pH 7,4) %1 SDS
3	Low-TE Buffer (pH 8,0)	10 mM Tris (pH 8,0) 0,1 mM EDTA (pH 8,0) 4 M Urea
	Isolation with Kit	
4	Tissue Lysis Buffer (pH 7.4)	200 mM Tris 20 mM NaCl 200 mM EDTA
5	Binding Buffer (pH 4.4)	6 M Guaninidine-HCl 10 mM Urea 10 mM Tris-HCl 20% Triton X-100 (v/v)
6	Inhibitor Removal Buffer (pH 6.6)	20 ml Absolute ethanol 5 M Guanidine-HCl 20 mM Tris-HCl
7	Wash Buffer (pH 7.5)	80 ml Absolute ethanol 20 mM NaCl 2 mM Tris-HCl
8	Elution Buffer (pH 8.5)	10 mM Tris-HCl
	Electrophoresis	
9	5X Tris Borate EDTA (TBE) Buffer (pH 8,3)	89 mM Tris-Base 89 mM Boric Acid 2 mM $Na_2EDTA.2H_2O$ (pH 8,3)
10	6X Loading Dye	10 mM Tris-HCl (pH 7.6) 0.03% bromophenol blue 0.03% xylene cyanol FF 60% glycerol 60 mM EDTA
11	Etidium Bromide (EtBr)	10 mg/ml
	PCR	
12	10X Taq Polymerase Buffer	200 mM $(NH4)_2SO_4$ 750 mM Tris-HCl (pH 8,8) % 0,1 Tween 20
13	MgCl2	25 mM MgCl2
14	dNTP mix	100 mM dATP, dCTP, dGTP & dTTP
15	Taq Polymerase	5 U / µl
16	Sterile dH_2O	Sterile Distilled Water

2.2 Methods

Manual DNA isolation was performed for collected tissue samples. In case the DNA isolation not achieved manually, isolation with kit is employed.

2.2.1 DNA Isolation

2.2.1.1 Manual DNA Isolation

DNA was extracted from a piece of muscle or skin tissue by the standard NaCl proteinase K procedure (Blin & Stafford, 1976).

Approximately 10 mg of tissue was ground and put in a 1.5 mL microcentrifuge tube containing 250 µl tissue SDS-lysis buffer and 10 µl proteinase K. 15-20 grains of chelex were added to tube and the samples were incubated for 1 to 3 hours at 56°C until the tissue was digested completely. Following the digestion, the tubes were centrifuged at 14500 rpm for 2 minutes. Supernatant was transferred to a new microcentrifuge tube and 100 µl NaCl (5M) and 100 µl dH_2O were added. After the tubes were mixed by inversion, additional centrifugation was employed for 10 minutes at 14500 rpm. Later on, supernatant was transferred to a new microcentrifuge tube and cold absolute ethanol (-20°C) 2.5 times their volume was added. After a gentle inversion step, tubes were centrifuged for an additional 10 minutes at 14500 rpm after which the supernatant was disposed. Afterwards, 200 µl of 70% ethanol was added to the tubes and centrifuged for 5 minutes at 14500 rpm. The supernatant was again disposed and the tubes stood in room temperature uncapped until they got dry. Finally, 50 µl lowTE was added to the tubes which were centrifuged for another 10-12 seconds. The microcentrifuge tubes containing extracted DNA were stored at -20°C for later analysis.

2.2.1.2 DNA Isolation with Kit

DNA was extracted from a piece of muscle or skin tissue, with the High Pure PCR Template Preparation Kit (Roche) following the manufacturer's protocol (Catalog Number 11 796 828 001).

Approximately 10 mg of tissue was ground and put in a 1.5 mL microcentrifuge tube containing 200 µl tissue lysis buffer and 20 µl proteinase K. The samples were incubated for 1 to 2 h at 55°C until the tissue was digested completely. 200 µl binding buffer was added and the tubes were incubated for 10 min at 70°C. After the addition of 100 µl isopropanol, samples were transferred to filter tubes and centrifuged for 1 min at 14,000 rpm. Later centrifugation flowthrough liquid was removed. 500 µl inhibitor removal buffer was added to the filter tubes which were centrifuged for 1 min at 14,000 rpm. After removing the flowthrough liquid, 500 µl wash buffer was added and the tubes were centrifuged for 1 min at 14,000 rpm. After discarding the flowthrough liquid, an additional 500 µl wash buffer was added and the tubes were centrifuged for 1 min at 14,000 rpm. After discarding the flowthrough liquid, the High Pure assembly was centrifuged for an additional 10s at 14,000 rpm to ensure removal of any residual wash buffer. To elute the DNA, filter tubes were inserted into a clean sterile 1.5 ml microcentrifuge tube. Prewarmed (70°C) 200 µl elution buffer was added and the tubes were centrifuged for another 1 min at 14,000 rpm. The microcentrifuge tubes containing the eluted DNA were stored at -20°C for later analysis.

2.2.1.3 Checking the Presence of DNA

After the isolation process, the presence of DNA was checked by agarose gel electrophoresis (MultiSub Midi with power supply EPS 301). 0.7% agarose gel was prepared by boiling agarose in 0.5 X TBE buffer. Following the boiling of the agarose gel, ethidium bromide (Et-Br), which is fluorescent under UV light when intercalated into DNA or RNA, was added to the solution with a final concentration of 0.5 µg/ml. The solution with Et-Br was poured into an agarose plate and stood

approximately 1 hour in room temperature for polymerization. The agarose plate was placed into the electrophoresis tank containing 0.5 X TBE buffer. DNA samples were prepared for loading by mixing 3 µl isolated DNA with 1 µl, 6X loading buffer (bromophenol blue dye) and loaded into wells of the gel. Also 2 µl of 1 kb DNA ladder was loaded into a well to compare the magnitude of isolated DNA fragments. The gel was run at 120 V and 300 mA for 15 minutes. After electrophoresis, the gel was transferred to a gel image system under UV for visualization. Concentrations of DNA samples were determined by comparing DNA band width with marker band width. No additional DNA quantity analysis was employed. Samples which gave thick bands were diluted with lowTE.

2.2.2 PCR

mtDNA DLoop, 16S rDNA and COI regions were amplified by using Polymerase Chain Reaction (PCR), using a thermo cycler (Techne TC-512). PCR technique can be summarised as described in Palumbi *et al.* (2002). It is a technique based on three steps which are called denaturation, annealing and extension respectively. In the denaturation step, disrupted hydrogen bonds between complementary bases yield single-stranded DNA molecules. In the annealing step, primers anneal to the single-stranded DNA template with hydrogen bonds and polymerase binds to the primer-template hybrid and begins the DNA synthesis. At the elongation step, DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the extending DNA strand.

2.2.2.1 Amplification of mtDNA Dloop

The 5' hypervariable portion of the mitochondrial Dloop (also known as control region) was amplified by PCR. H00034 (Rosel *et al.*, 1995) and D_Loop16L (Hoelzel *et al.*, 1991) primers were employed to synthesize the partial D-loop of mtDNA. A total of 51 samples were examined and the length of the aligned mtDNA Dloop

sequences was 607 base pairs. 48 of the 51 samples were *Phocoena phocoena*, 1 of them were *Stenella coerualba* and 2 of them were *Tursiops truncatus*. PCR reaction mix contents and PCR conditions for H00034 & D_Loop16L primers are given below in Table 2.3.

Table2.3.mtDNA Dloop PCR reaction mix contents and PCR conditions for H00034 & D_Loop16 L primers

PCR reaction mix contents			
Reagents	**Volume needed per reaction**	**Concentration**	
Template DNA	1.0 µl		
dNTP mix	0.2 µl	25 mM (for each nucleotide)	
10X Buffer	2.5 µl	10 X	
MgCl2	2.0 µl	25 mM	
Primer H00034	0.1 µl	50 µM	
Primer D_Loop16L	0.1 µl	50 µM	
Taq Polymerase	0.2 µl	5 U/µl	
Distilled Water	19.4 µl	N/A	
Total	25.0 µl		
PCR conditions			
PCR Step	**Number of cycles**	**Temperature**	**Time**
1. Initial denaturation	1	94 °C	2 min
2. Denaturation	35	94 °C	30 s
3. Annealing	35	52 °C	30 s
4. Extension	35	72 °C	1 min
5. Final extension	1	72 °C	5 min

Primers used for mtDNA Control Region amplification is given below.

H00034 : 5'-TACCAATGTATGAAACCTCAG-3'

D_Loop16 L : 5'-CCCGGTCTGTAAACC -3'

2.2.2.2 Amplification of mtDNA 16S rDNA

The partial mitochondrial 16S rDNA was amplified by PCR. 16Sar_L and 16Sbr_H primers (Palumbi *et al.* 2002) were employed to synthesize the partial 16S rDNA of mtDNA. A total of 48 samples were examined and the length of the aligned mtDNA 16S rDNA sequences was 532 base pairs. 44 of the 48 samples were *Phocoena phocoena,* 3 of them were *Tursiops truncatus* and 1 of them was *Stenella*

coerualba. PCR reaction mix contents and PCR conditions for 16Sar_L & 16Sbr_H primers are given below in Table 2.4.

Table2.4 mtDNA 16S PCR reaction mix contents and PCR conditions for 16Sar_L & 16Sbr_H primers

PCR reaction mix contents			
Reagents	**Volume needed per reaction**	**Concentration**	
Template DNA	1.0 µl		
dNTP mix	0.2 µl	25 mM (for each nucleotide)	
10X Buffer	2.5 µl	10 X	
MgCl2	1.5 µl	25 mM	
Primer H00034	0.1 µl	50 µM	
Primer D_Loop16L	0.1 µl	50 µM	
Taq Polymerase	0.2 µl	5 U/µl	
Distilled Water	19.4 µl	N/A	
Total	25.0 µl		
PCR conditions			
PCR Step	**Number of cycles**	**Temperature**	**Time**
1. Initial denaturation	1	94 °C	2 min
2. Denaturation	40	94 °C	30 s
3. Annealing	40	50 °C	30 s
4. Extension	40	72 °C	1 min
5. Final extension	1	72 °C	5 min

Primers used for 16S rDNA amplification is given below.

16Sar_L : 5'-CGCCTGTTTATCAAAAACAT-3'

16Sbr_H : 5'-CCGGTCTGAACTCAGATCACGT-3'

2.2.2.3 Amplification of mtDNA COI

The partial mitochondrial mtDNA COI was amplified by PCR. COIfishF1 and COIfishR1 primers (Ward *et al., 2005)* were employed to synthesize the partial mtDNA COI. A total of 44 samples were examined and the length of the aligned mtDNA COI sequences was 549 base pairs. 44 of the samples were *Phocoena phocoena.* PCR reaction mix contents and PCR conditions for COIfishF1 & COIfishR1 primers are given below in Table 2.5.

Table 2.5 mtDNA COI PCR reaction mix contents and PCR conditions for COIfishF1 & COIfishR1 primers

PCR reaction mix contents			
Reagents	**Volume needed per reaction**	**Concentration**	
Template DNA	1.0 µl		
dNTP mix	0.2 µl	25 mM (for each nucleotide)	
10X Buffer	2.5 µl	10 X	
MgCl2	1.5 µl	25 mM	
Primer H00034	0.1 µl	50 µM	
Primer D_Loop16L	0.1 µl	50 µM	
Taq Polymerase	0.2 µl	5 U/µl	
Distilled Water	19.4 µl	N/A	
Total	25.0 µl		
PCR conditions			
PCR Step	**Number of cycles**	**Temperature**	**Time**
1. Initial denaturation	1	94 °C	2 min
2. Denaturation	35	94 °C	30 s
3. Annealing	35	54 °C	30 s
4. Extension	35	72 °C	1 min
5. Final extension	1	72 °C	5 min

Primers used for mtDNA COI amplification is given below.

COIfishF1 : 5'-TCAACCAACCACAAAGACATTGGCAC-3'

COIfishR1 : 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'

2.2.2.4 Checking the Presence of PCR Products

The results of the PCR amplification was checked by the visualization of the agarose gel electrophoresis. For mtDNA Dloop, 16S rDNA and COI region PCR products, 1% agarose gel was prepared by boiling agarose in 0.5 X TBE buffer. After boiling the agarose gel, ethidium bromide (Et-Br) was added to the solution with a final concentration of 0.5 µg /ml. Then the solution was poured into an agarose plate and stood approximately 1 hour at room temperature for polymerization. The agarose plate was placed into the electrophoresis tank containing 0.5 X TBE buffer. DNA samples were prepared for loading by mixing 5 µl PCR product with 1 µl, 6X loading buffer (bromophenol blue dye) and loaded into wells of the gel. Also 2 µl of

100bp DNA ladder was loaded into a well to compare the magnitude of PCR products. The gel was visualized under UV light by a gel image system following electrophoresis at 120 V and 300 mA for 15 minutes.

Concentrations of PCR products were determined by comparing DNA band width with marker band width. Whenever PCR bands widths were weak PCR repeated with 2 µl of template DNA. DNA samples which failed to give PCR products were subject to one more additional ethanol precipitation step, before repeating PCR. Absolute ethanol, 2.5 times volume of the DNA sample, was added into a microcentrifuge tube and gentle inversion was employed. Centrifugation was done at 14500 rpm for 20 minutes. Afterwards, the supernatant was discarded and the tubes stood at room temperature, until all the alcohol evaporated. LowTE was added equal to the initial volume of the DNA samples. PCR was repeated with these DNA samples.

Although this additional ethanol precipitation step provided better PCR products for some samples, it did not work for all the samples. The samples which failed to give PCR products after the additional ethanol precipitation step was exposed to another additional purification procedure with the High Pure PCR Template Preparation Kit and PCR was repeated. Except for adding Proteinase K and the incubation step for 1 to 2 h at 55°C, all the steps in DNA isolation with kit were employed (Described in section DNA Isolation with Kit). Although Proteinase K helps the tissue become digested, we had already had isolated DNA in microcentrifuge tubes. Hence, the addition of Proteinase K and incubation were skipped.

In spite of all performed procedures, some of the samples failed to give COI and/or 16S and/or Dloop fragments. These samples excluded from following analysis.

2.2.2.5 Purification of PCR Products

Before the sequence analysis of COI, 16S rDNA and Dloop of mtDNA, PCR products were purified with the High Pure PCR Product Purification Kit (Roche) according to the manufacturer's protocol (Catalog Number 11 732 676 001).

500 µl of binding buffer was added to 100 µl of PCR product in microcentrifuge tubes and mixed vigorously. A high pure filter tube was inserted into the collection tube and the samples were transferred into the upper reservoir of those filter tubes. Following 30 seconds of centrifugation at 14500 rpm, the flowthrough solution was discarded and 500 µl of wash buffer was added again to the upper reservoir of the filter tubes. Another centrifugation was performed for 1 minute at 14500 rpm and the flowthrough solution was discarded. An additional 200 µl of wash buffer was added to the filter tubes, centrifuged for 1 minute at 14500 rpm and the flowthrough solution was discarded with collection tubes. One last washing step was performed to ensure optimal purity and the complete removal of wash buffer from the glass fibers. Filter tubes were reconnected to clean 1.5 ml microcentrifuge tubes. 50 µl elution buffer were added to the filter tubes which were centrifuged at 14500 rpm for 1 minute. The microcentrifuge tubes containing purified DNA were stored at -20°C for later analysis.

2.2.3 Sequence Analysis of mtDNA DLoop, 16S rDNA and COI

Before purified PCR products were sent to Macrogen Inc. Seoul, Korea for sequence analysis, they were checked on 1% agarose gel in order to verify PCR products. Cycle sequencing was performed using 80-100 ng of purified PCR product with ABI Prism BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and PCR primers. Band separation was carried out on an ABI PRISM 377 Automated Sequencer (Applied Biosystems). COIfishF1, 16Sar-L and D_Loop16L primers were used respectively for sequencing COI, 16S rDNA and Dloop of mtDNA.

2.2.4. Sequence Alignment and Phylogenetic Analysis

Received raw sequence chromatograms were corrected by eye in Chromas pro (Technelysium Pty Ltd) and the low quality sequences were trimmed from both ends and the sequence data were saved in FASTA format for later analysis.

For sequence and phylogenetic analysis additional 38 *P.p*, 3 *T.t* and 3 *S.c* sequences obtained from Gen-Bank were also included to improve phylogenetic accuracy. List of the sequences retrieved from Gen-Bank can be seen in Table 2.6.

Table 2.6 List of sequences retrieved from Gen-Bank

Accession no	Haplotype Name	Location	Reference
P.p			
EF063646	I	5B 17T 6G40U 5AG	Viard-Martinez et al. 2007
EF063647	II	1B	Viard-Martinez et al. 2007
EF063648	III	1B	Viard-Martinez et al. 2007
EF063649	IV	2B 1T	Viard-Martinez et al. 2007
EF063650	V	1T	Viard-Martinez et al. 2007
EF063651	VI	1T	Viard-Martinez et al. 2007
EF063652	VII	1T	Viard-Martinez et al. 2007
EF063653	VIII	1T 2G40U	Viard-Martinez et al. 2007
EF063654	IX	1T 1U	Viard-Martinez et al. 2007
EF063655	X	1T 3U	Viard-Martinez et al. 2007
EF063656	XI	2T 3U	Viard-Martinez et al. 2007
EF063657	XII	1U	Viard-Martinez et al. 2007
EF063658	XIII	1U	Viard-Martinez et al. 2007
EF063659	XIV	1U	Viard-Martinez et al. 2007
EF063660	XV	1U	Viard-Martinez et al. 2007
EF063661	XVI	1U	Viard-Martinez et al. 2007
EF063662	XVII	2U	Viard-Martinez et al. 2007
EF063663	XVIII	3M	Viard-Martinez et al. 2007
EF063664	XIX	1A	Viard-Martinez et al. 2007
EF063665	XX	1A	Viard-Martinez et al. 2007
EF063666	XXI	1A	Viard-Martinez et al. 2007
EF063667	XXII	1A	Viard-Martinez et al. 2007
EF063668	XXIII	1A	Viard-Martinez et al. 2007
EF063669	XXIV	1A	Viard-Martinez et al. 2007
EF063670	XXV	1A	Viard-Martinez et al. 2007
EF063671	XXVI	2A	Viard-Martinez et al. 2007
EF063672	XXVII	3A	Viard-Martinez et al. 2007
EF063673	XXVIII	10A	Viard-Martinez et al. 2007
EF063674	XXIX	16A	Viard-Martinez et al. 2007
EF063675	XXX	1Gb	Viard-Martinez et al. 2007
EF063676	XXXI	3Gb	Viard-Martinez et al. 2007
EF063110	XXXII	1AG	Viard-Martinez et al. 2007
U09689		T	Rand et al., 1995
U09690		T	Rand et al., 1995
U09691		T	Rand et al., 1995
HM130290			Zhang et al., unpublished
HM130292			Zhang et al., unpublished
NC_005280	(Complete mitochondrial genome)		Arnason et al., 2004
T.t			
AY968608		WM	Nirdi et al., 2005
AY968599		BM	Nirdi et al., 2005
NC_012959	(Complete mitochondrial genome)		Xiao et al., 2009
S.c			
M38497			McGraw et al., 2008
AH10816			Dujon et al., unpublished
NC_012953	(Complete mitochondrial genome)		Xiao et al., 2009

Multiple sequence alignment was run by ClustalW in Mega version 5 (Tamura, *et al.,* 2011). After multiple alignment identical sequences were removed from the alignment and only variable sites were used for phylogenetic analysis.

Optimal model for sequence evolution for the likelihood analysis, which is fundamental to statistical phylogenetic inference, was determined with MrModeltest 2 (Nylander, 2004) using hierarchical likelihood ratio tests. The use of statistical approaches to select an appropriate model of sequence evolution for phylogenetic inference is well-established and built on a robust literature (Sullivan & Joyce, 2005). All model selection methods try to find "a best approximating model" that balances systematic and stochastic errors (Burnham & Anderson, 2003).

Recommended sequence evolution models for Dloop and COI analysis by MrModeltest can be seen in Table 2.7.16S sequences were not subjected into phylogenetic analysis because of low genotype number. Because numerous sequences added to Dloop data set from Gen-Bank, phylogenetic analyses run for two different data sets. While one data set contains only collected samples' genotypes, the second data set contains both collected samples' genotypes and the haplotypes retrieved from Gen-Bank.

Table2.7 Best fit models for Dloop and COI sequences

Data Set	Best Fit Model	Program Settings for PAUP	Program Settings for Mr.Bayes
Dloop Genotypes 1-20	(HKY+I+G)	Lset Base=(0.3147 0.2632 0.1309) Nst=2 TRatio=9.1697 Rates=gamma Shape=0.8945 Pinvar=0.8805;	Lset nst=2 rates=invgamma; Prset statefreqpr=dirichlet(1,1,1,1);
Dloop Genotypes 1-20 & Genebank Haplotypes	(HKY+G)	Lset Base=(0.3155 0.2588 0.1253) Nst=2 TRatio=15.2610 Rates=gamma Shape=0.0087 Pinvar=0;	Lset nst=2 rates=gamma; Prset statefreqpr=dirichlet(1,1,1,1);
COI_ Genotypes 1-8 & Genebank Haplotypes	K80	Lset Base=equal Nst=2 TRatio=18.1482 Rates=equal Pinvar=0;	Lset nst=2 rates=equal; Prset statefreqpr=fixed(equal);

For phylogenetic reconstruction, we used the maximum parsimony (MP) method and maximum likelihood (ML) method implemented in PAUP* 4.0b10 (Swofford, 2002) and a bayesian Inference approach (BI) implemented using the programme MrBayes 3.1 (Huelsenbeck & Ronquist, 2001;Ronquist & Huelsenbeck, 2003).

The maximum parsimony analysis, a heuristic search of 10 random additions with tree-bisection-reconnection (TBR) branch swapping was performed with MULPAR and steepest descent options. For the phylogenetic reconstruction based on a bayesian approach, the number of generations for the Monte Carlo Markov chains (MCMC) method was set to 200,000 and a tree was saved every ten generations. The burnin value used in the MCMC chains was set to 5,000. The consensus tree was produced using PAUP retaining branches with 50% support or greater. Haplotypes added to second Dloop data set, which were retrieved from Gen-Bank, were relatively shorter compare to genotype sequences found in this study. The missing parts of the sequences were defined to PAUP as missing data to prevent interpretation of the data as deletion. An example of nexus file for parsimony analysis of COI can be seen in Figure 2.2.

```
#nexus

begin paup;
set autoclose
nowarntree
nowarnreset
defaultmode;

log file=COI_genotip.log;
execute COI_genotip.nex;

outgroup NC_005280;

set criterion=parsimony;
hsearch nreps=10000 addseq=random swap=tbr
rearrlimit=100000 limitperrep=yes;
savetrees file=COI_genotip_mp.tre brlens;
gettrees allblocks=yes duptrees=keep
storetreewts=yes mode=7 file=COI_genotip_mp.tre;
log file=parsimonyconsensus.log;
contree /majrule=yes strict=no le50=yes
showtree=yes treefile=COI_genotip_MPconsensus.tre
grpfreq=yes;

gettrees
     mode=3
     file=COI_genotip_MPconsensus.tre;
roottrees;
showdist;
describetrees /plot=phylogram brlens=yes
labelnode=no;
describetrees /plot=cladogram brlens=yes;

log stop;
quit;
end;
```

Figure 2.2 The nexus block that is used for parsimony analysis in PAUP

CHAPTER THREE
RESULTS

3.1 Results of Laboratory Studies

3.1.1 Checking the Presence and Quality of DNA

After the isolation processes described in materials and methods, DNA extractions were checked to visualise DNA presence and quality as in described in materials and methods. An example of an agarose gel image of DNA scanned under UV light can be seen in Figure 3.1 and 3.2.

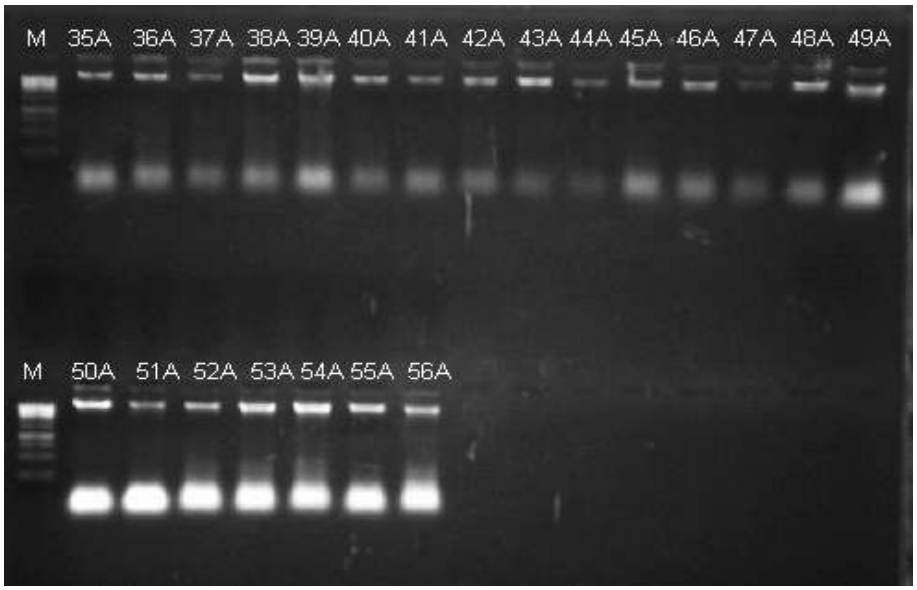

Figure 3.1 0.7% agarose gel image of total DNA extraction after manual isolation

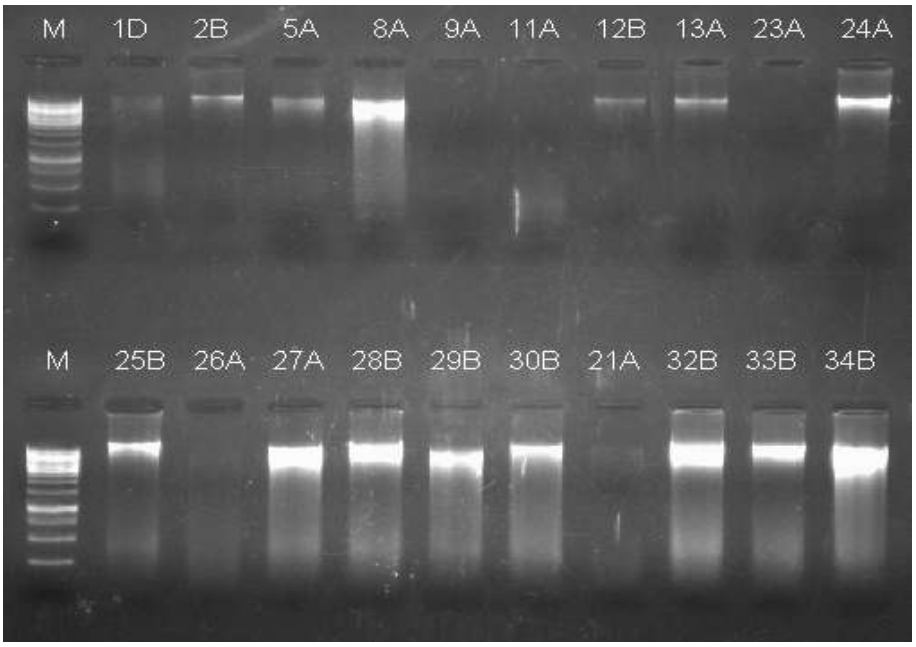

Figure 3.2 0.7% agarose gel image of total DNA extraction after isolation with the High Pure PCR Template Preparation Kit

3.1.2 Checking the PCR Products

After PCR processes described in materials and methods, PCR products were checked to visualise PCR product presence and quality as described in materials and methods.

3.1.2.1 Dloop PCR Products

The majority of the samples did not give Dloop PCR products in the beginning. The DNA samples which failed to give PCR products were exposed to an additional ethanol precipitation step as described in materials and methods section and PCR was repeated. An example of an agarose gel image of PCR products scanned under UV light can be seen in Figure 3.3 and 3.4.

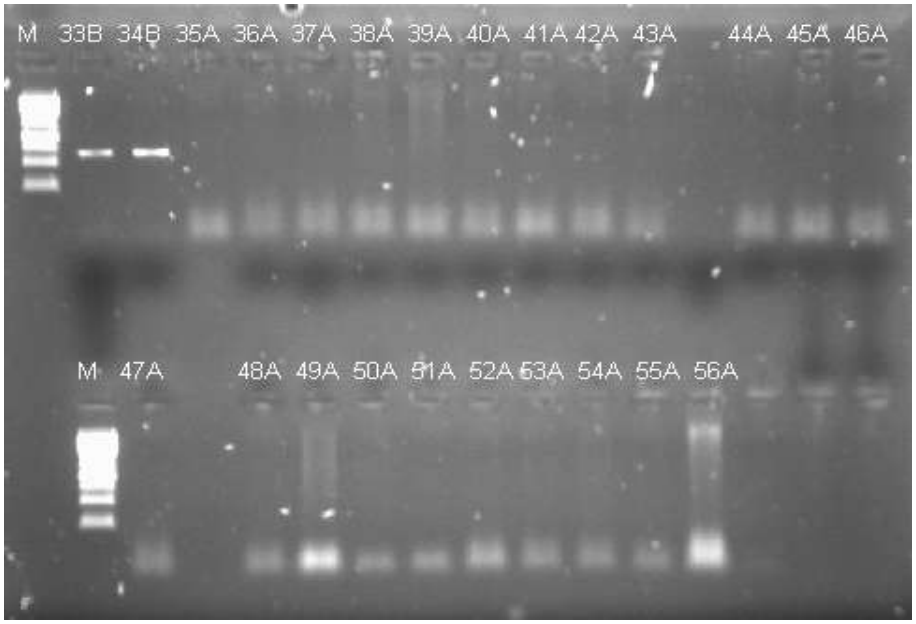

Figure 3.3 1% agarose gel image of samples which failed to give Dloop PCR products

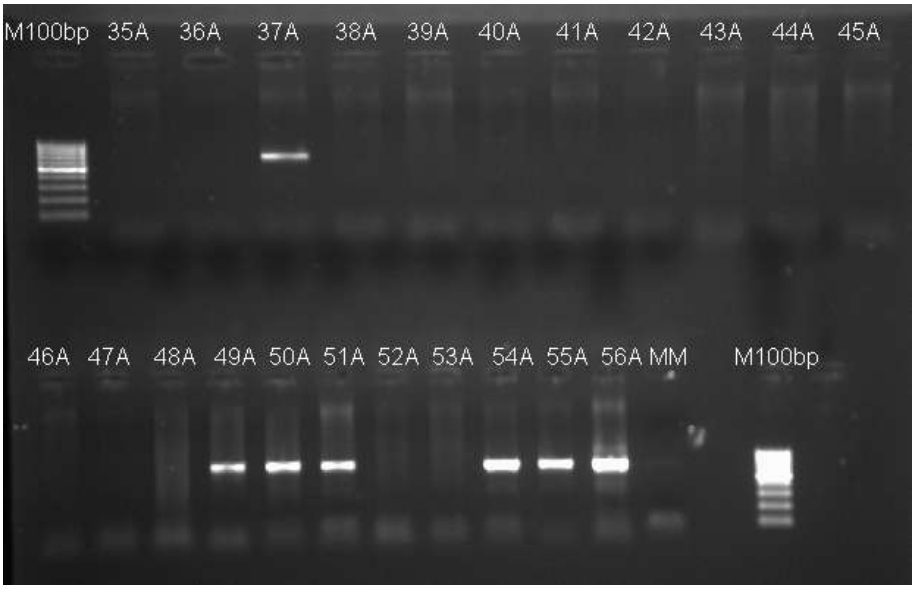

Figure 3.4 1% agarose gel image of Dloop PCR after applying an additional ethanol precipitation step

Although the additional ethanol precipitation step was applied, the samples did not give PCR products afterwards. These samples were exposed to another additional purification step as described in materials and methods and Dloop PCR was achieved again after this second purification step. An example of an agarose gel image of PCR

products after applying a second additional purification step can be seen in Figure 3.5.

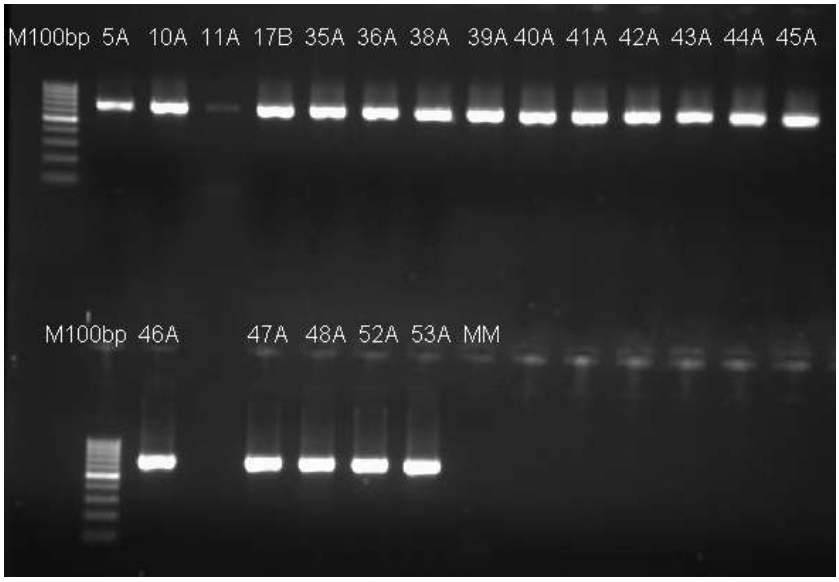

Figure 3.5 1% gel image of Dloop PCR after applying a second additional purification step

3.1.2.2 16sDNA PCR Products

The majority of the samples did give 16sDNA PCR products in the beginning. The DNA samples which failed to give PCR products were exposed to an additional ethanol precipitation step and PCR was repeated. An example of an agarose gel image of PCR products scanned under UV light can be seen in Figure 3.6 and 3.7.

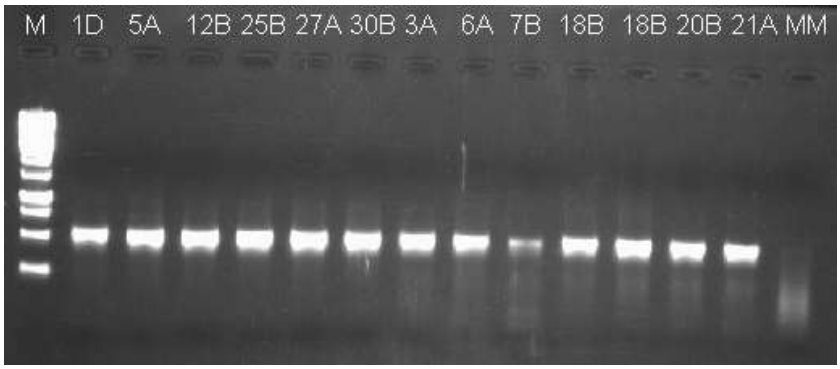

Figure 3.6 1% agarose gel image of 16sDNA PCR products

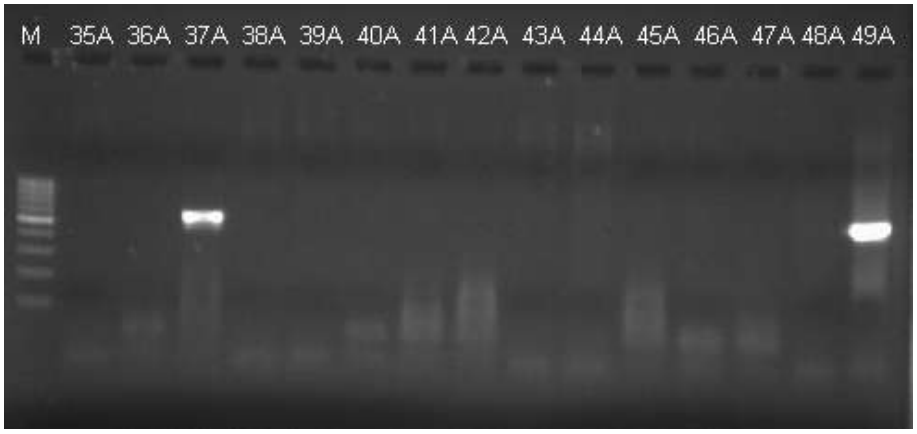

Figure 3.7 1% agarose gel image of samples which failed to give 16sDNA PCR products

Although the additional ethanol precipitation step was applied, the samples did not give PCR products afterwards. These samples were exposed to another additional purification step as described in materials and methods and 16sDNA PCR was achieved again after this second purification step. An example of an agarose gel image of PCR products scanned under UV light can be seen in Figure 3.8 and 3.9.

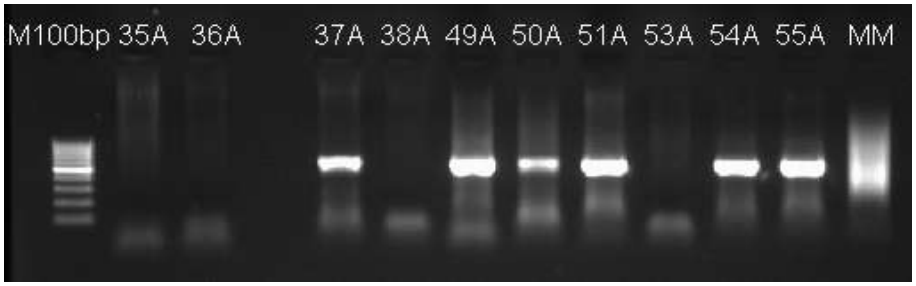

Figure 3.8 1% gel image of 16sDNA PCR after applying an additional ethanol precipitation step

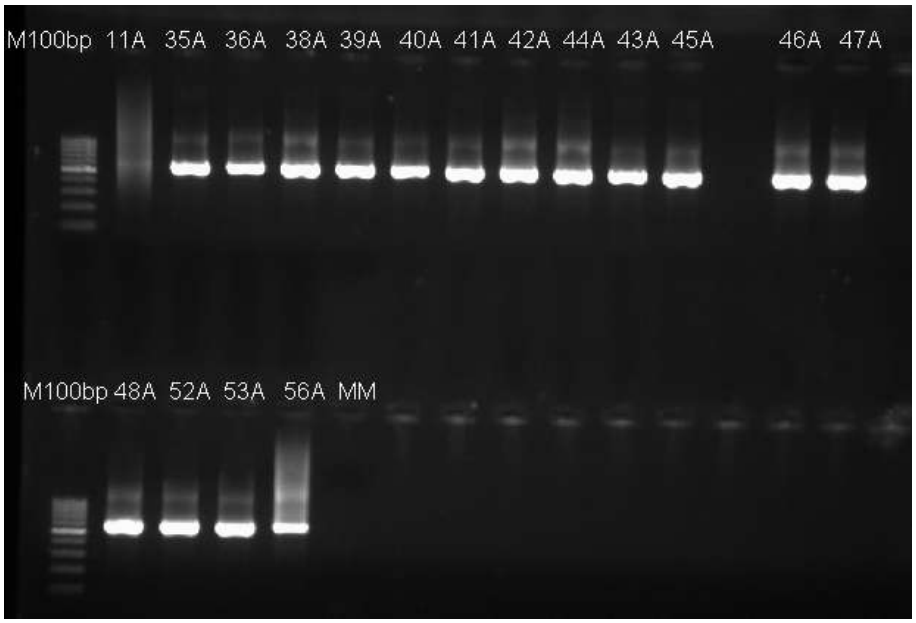

Figure 3.9 1% gel image of 16sDNA PCR after applying a second additional purification step

3.1.2.3 COI PCR Products

The majority of the samples did not give COI PCR products in the beginning. The DNA samples which failed to give PCR products were exposed to an additional ethanol precipitation step as described in materials and methods section and PCR was repeated. An example of an agarose gel image of PCR products scanned under UV light can be seen in Figure 3.10 and 3.11.

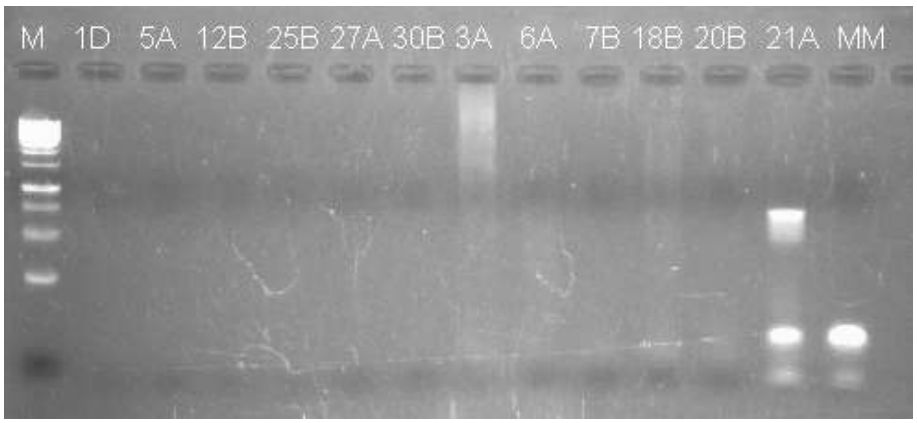

Figure 3.10 1% agarose gel image of samples which failed to give COI PCR products

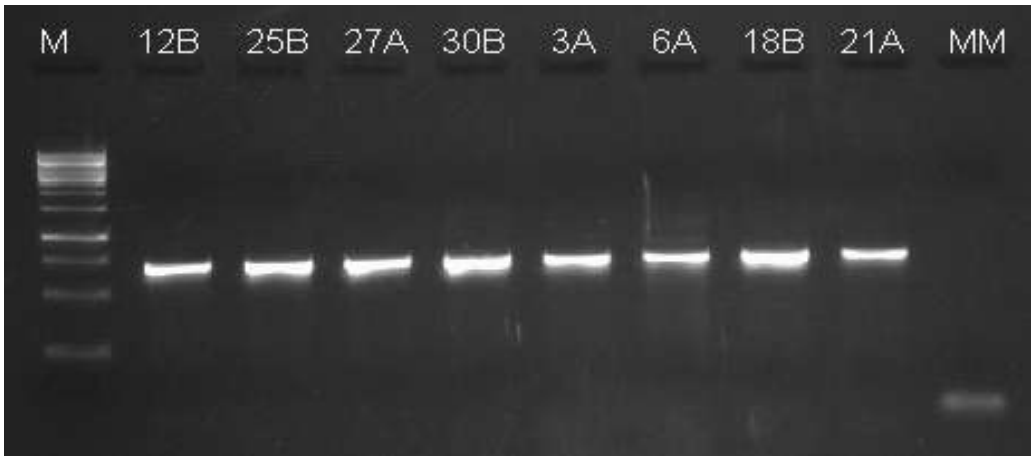

Figure 3.11 1% agarose gel image of COI PCR products after applying an additional ethanol precipitation step

Although the additional ethanol precipitation step was applied, some of the samples did not give PCR products afterwards. These samples were exposed to another additional purification step as described in materials and methods and COI PCR was achieved after this second purification. An example of an agarose gel image of PCR products scanned under UV light can be seen in Figure 3.12 and 3.13.

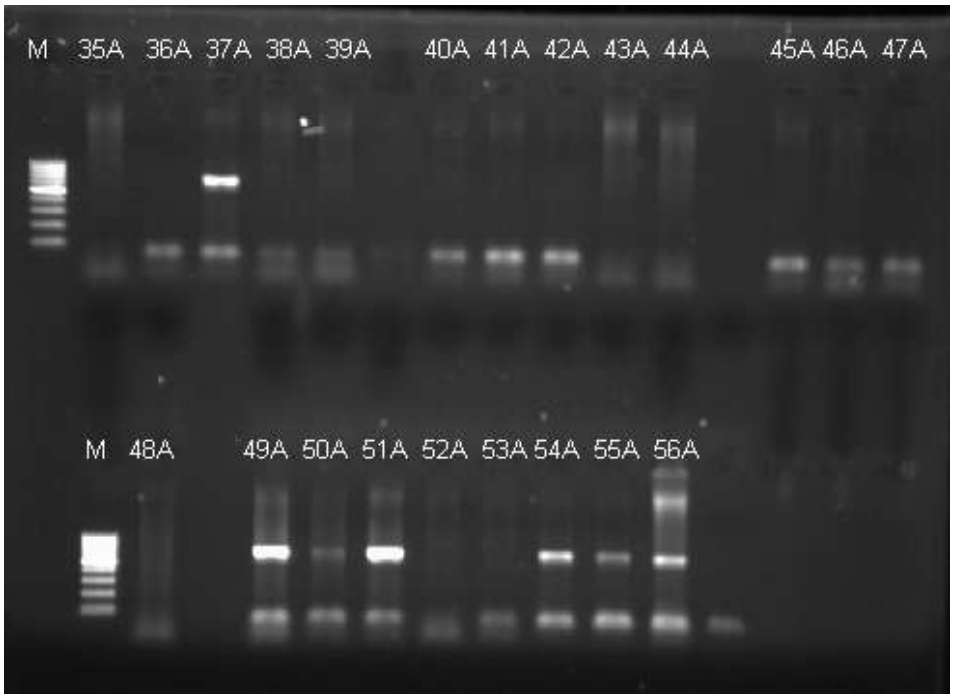

Figure 3.12 1% gel image of COI PCR after applying an additional ethanol precipitation step

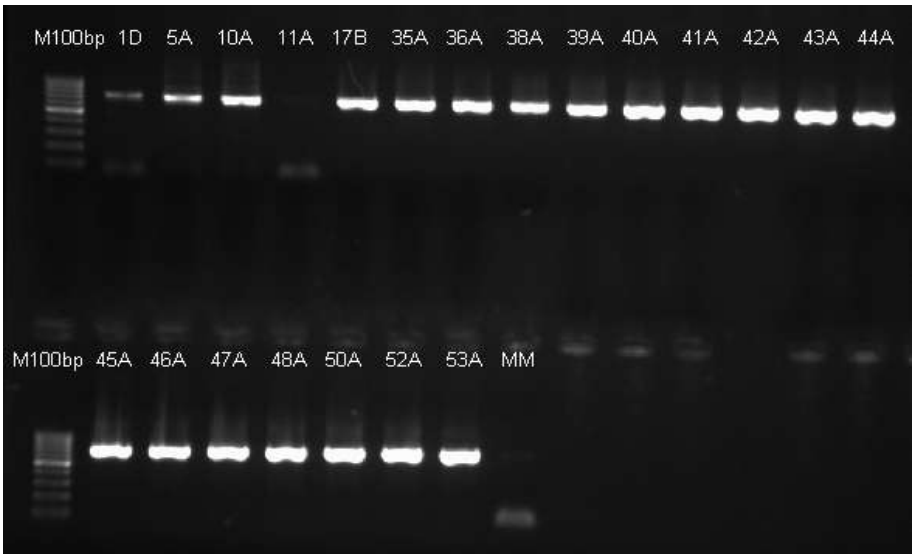

Figure 3.13 1% gel image of COI PCR after applying a second additional purification step

3.1.3 Purification of the PCR Products

The PCR product purification was accomplished mostly but a few PCR products of some samples were lost during the purification process. In that case, a new PCR was applied for these samples and purification repeated again. An example of an agarose gel image of purified PCR products scanned under UV light can be seen in Figure 3.14, 3.15, 3.16 and 3.17.

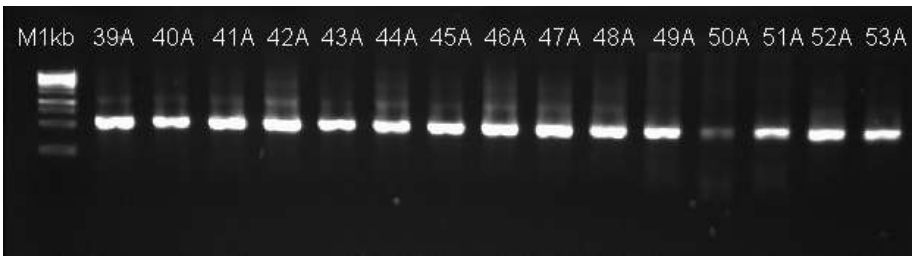

Figure 3.14 1% agarose gel image of 16SDNA PCR products purification

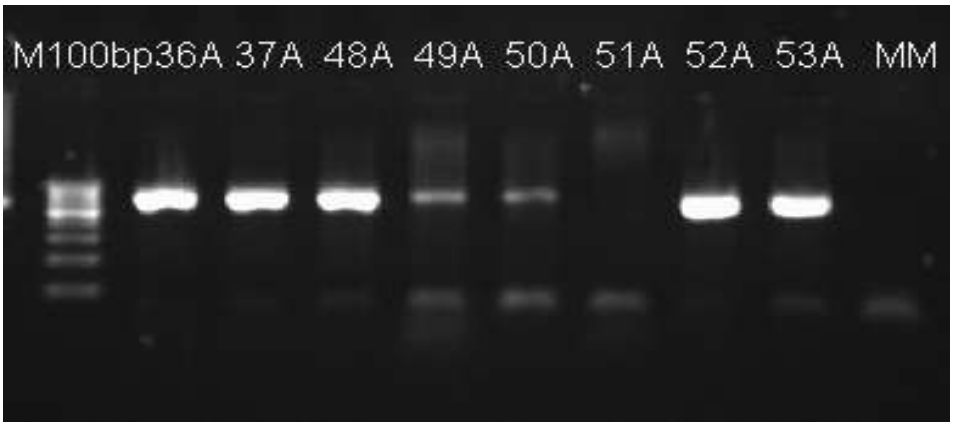

Figure 3.15 1% agarose gel image of COI PCR products purification

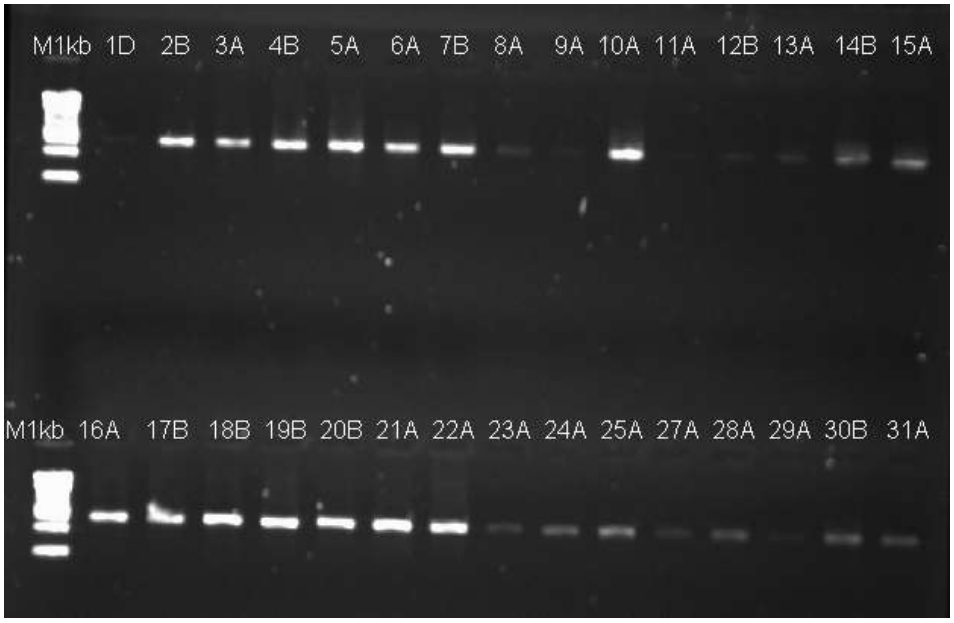

Figure 3.16 1% agarose gel image of Dloop PCR products purification

Figure 3.17 1% agarose gel image of Dloop and COI PCR products after a second PCR and purification

3.2 Results of Sequence and Phylogenetic Analysis

Received chromatograms of Dloop, 16S and COI sequences corrected by eye. A sample chromatogram of COI sequence and an example of a base substitution can be seen in Figure 3.18 and 3.19.

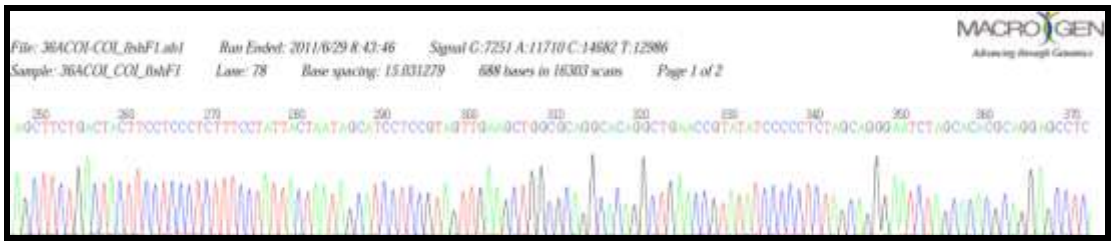

Figure 3.18 A sample from the chromatogram of the COI PCR product

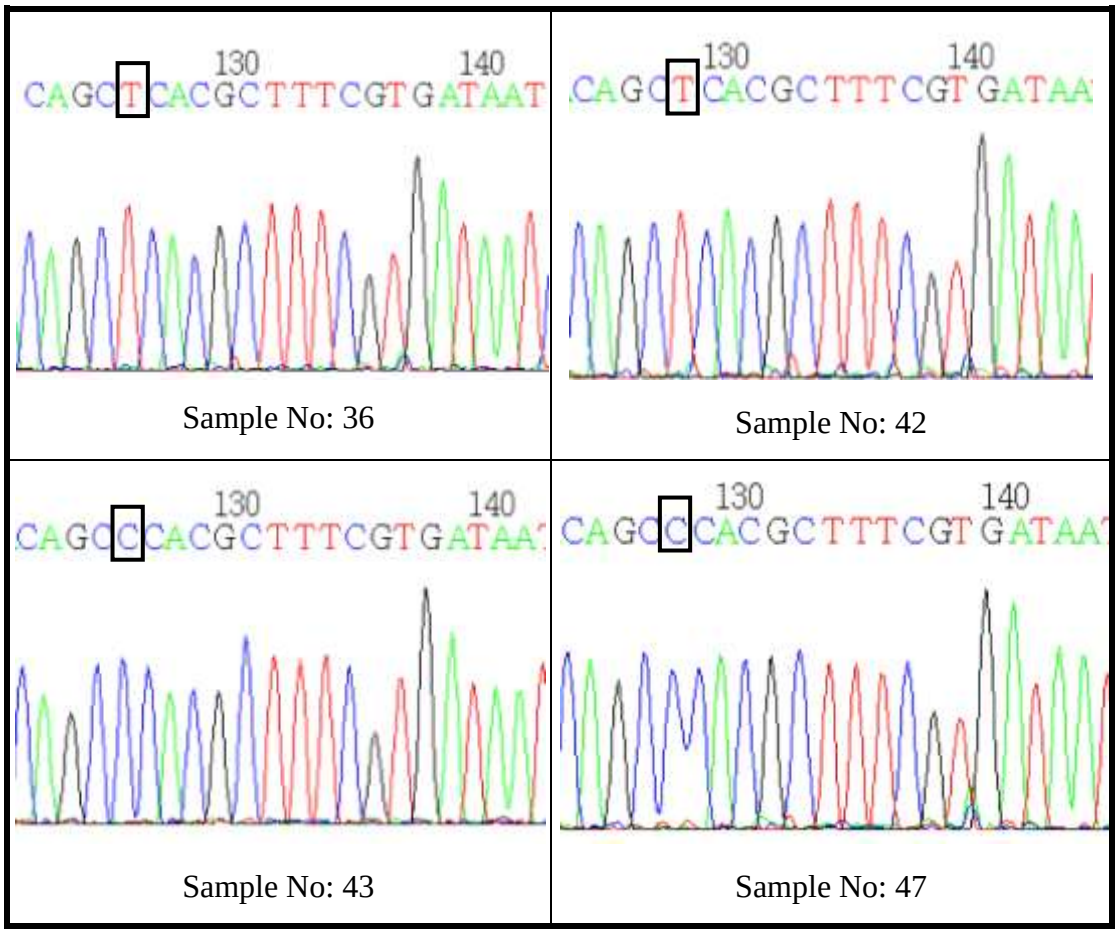

Figure 3.19 4 samples from the chromatograms of different individuals which show 2 genotypes in the mtDNA COI region

3.2.1 Dloop Results

A 599 bp fragment of Dloop was used for alignment from a total of 51 harbour porpoises. Our sequences align with the published U09691 sequence, starting from base position 45 up to 506. All sequences retrieved from Gen-Bank can be seen in materials and methods, Table 2.6.

20 genotypes were defined from 48 sampled harbour porpoises (47 Black Sea samples and 1 Aegean Sea sample) with 6 parsimony informative sites, 1 insertion and 17 base substitutions. 7 of these genotypes were shared between two or more individuals and the remaining 13 genotypes were unique. The most frequent genotype (Genotype 1) represented 43.8% of all individuals analyzed. The 13 unique genotypes had samples from 4 different locations. 7 of the unique genotypes came from Karaburun, 4 from Şile, 1 from Kıyıköy and 1 from Russia.

In addition to 20 genotypes found in this study, 3 more genotypes, U09689, U09690 and U09691, were added to the analysis from Gen-Bank to improve phylogenetic accuracy. Variable sites of aligned Dloop genotypes from 51 harbour porpoises and genotype affiliations of 48 collected samples can be seen in Figure 3.20 and Table 3.1.

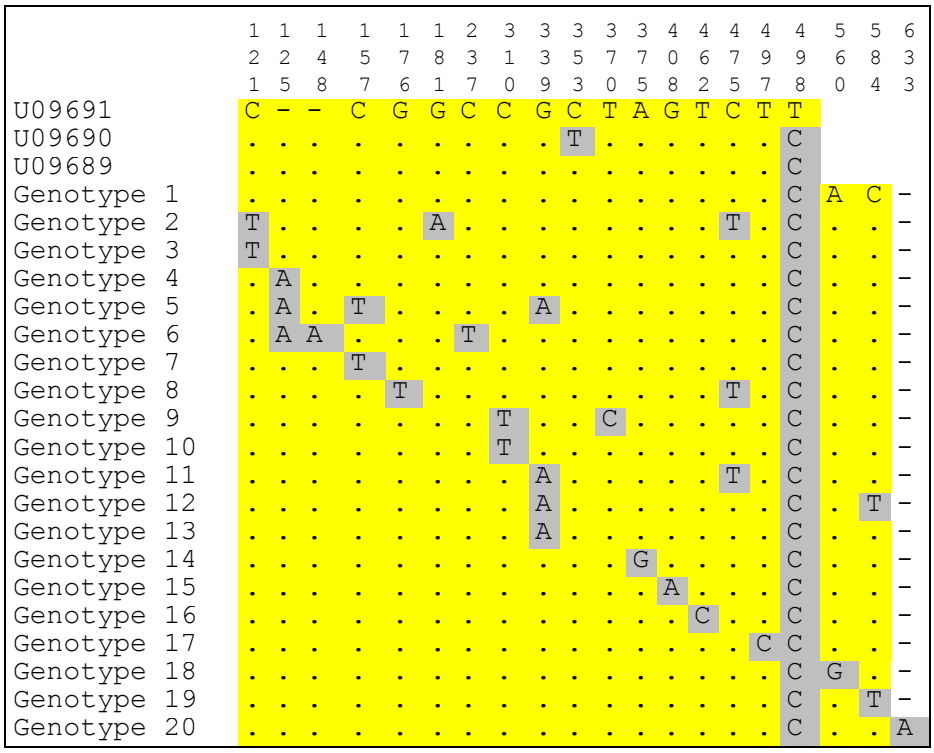

Figure 3.20 The variable sites in 23 genotypes identified for 47 Black Sea, 1 Aegean sample and 3 sample s retrieved from Gen-Bank. Numbered site refer to published *P.p* Dloop sequence U09691(Rosel *et al.*2005).

Table 3.1 Genotype affiliations of 48 collected samples

	Genotype 1		Genotype 2	Genotype 3	Genotype 4	Genotype 5	Genotype 6
Location	Karaburun (12) Kıyıköy (3) Ereğli (1) Zonguldak (1)	Fatsa (1) Şile (1) Rumeli Feneri (1) Romania (1)	Russia (1)	Sinop (1) Karaburun (1)	Karaburun (1) Kefken (1) Russia (1)	Karaburun (1)	Karaburun (1)
	Genotype 7	Genotype 8	Genotype 9	Genotype 10	Genotype 11	Genotype 12	Genotype 13
Location	Şile (1)	Kıyıköy (1)	Karaburun (1)	Rumeli Feneri (1) İğneada (1)	Şile (1)	Karaburun (1)	Karaburun (1)
	Genotype 14	Genotype 15	Genotype 16	Genotype 17	Genotype 18	Genotype 19	Genotype 20
Location	Karaburun (1)	Karaburun (1)	Karaburun (2)	Karaburun (2) Fatsa (1)	Şile (1)	Şile (1)	Urla (1) Zonguldak (1)

The phylogenetic analysis was achieved for two different data sets using three phylogenetic inference methods; maximum parsimony, maximum likelihood and bayesian inference. First data set consists of only Dloop genotypes collected from the 48 P.p samples and the second consists of both the collected samples' genotypes and 36 P.p haplotypes gathered from Gen-Bank.

The consensus tree given by MrBayes for Dloop genotypes of the collected 48 P.p samples didn't show any branch formations; therefore the tree is not represented here. The maximum likelihood consensus tree given by PAUP showed 4 distinct clades with 100% probability values. Three out of four of the clades had samples from Karaburun, Rumeli Feneri, İğneada and Şile, which are the most closely neighbouring sampling sites in the study. The remaining clade had samples in relatively more dispersed geographical locations such as Russia, Sinop and Karaburun. The maximum likelihood consensus tree for Dloop genotypes can be seen in Figure 1 in Appendix 1. Lastly, the maximum parsimony consensus tree given by PAUP showed 1 clade formation with 100% probability value. The clade consists of samples from geographically closed locations, like Karaburun, Rumeli Feneri and İğneada. The maximum parsimony consensus tree for this Dloop data set can be seen in Figure 2 in Appendix 1.

The only clade formation which was supported by two different methods, ML and MP, consisted two genotypes; Genotype 9 and Genotype 10 with samples from Karaburun, Rumeli Feneri and İğneada.

The bayesian consensus trees given by MrBayes for the second Dloop data set of all collected samples' genotypes and the haplotypes gathered from Gen-Bank, showed 7 clades. Numbers on the branches gives the probability of each partition or clade in the tree. The clade formation with the highest probability (%100) value was observed for the Atlantic samples. The Black Sea samples which were found in same clades were from geographically widespread locations. None of the samples from same location formed distinct clades. The bayesian consensus trees given by MrBayes for the second Dloop data set can be seen in Figure 3 in Appendix 1. The

maximum likelihood consensus tree showed 11 distinct clades with 100% probability values. 10 of the 11 clades were from geographically widespread locations. But 1 clade had samples from relatively close locations, Karaburun, Şile and Bulgaria. None of the samples from the exact same location formed distinct clades. Maximum likelihood consensus tree for all Dloop datas can be seen in Figure 4 in Appendix 1. The maximum parsimony consensus tree given by PAUP showed 6 clade formations with different probability values between 56% and 100%. One of the clades with 56% probability consists of samples from geographically closed locations, like Karaburun, Şile and Bulgaria which was also supported by maximum likelihood consensus tree with 100% probability. The maximum parsimony consensus tree for this second Dloop data set can be seen in Figure 5 in Appendix 1.

20 genotypes gathered from 48 collected P.p samples were aligned P.p haplotypes retrieved from Gen-Bank (Viaud-Martinez *et al.*, 2007). Retrieved haplotypes' detailed information can be found in materials and methods section Table 2.6. Fourteen of the genotypes matched with haplotypes, remaining 6 genotypes were new Black Sea harbour porpoise sequences. Because the retrieved haplotype sequences were 192 bp shorter than the genotype sequences, 7 different genotypes corresponded to one haplotype. Genotype-haplotype affiliations can be found in Table 3.2.

Genotypes 1, 2, 16, 17, 18, 19 and 20, which are possibly equivalent to Haplotype I, are geographically distributed among the Black Sea of Turkey, Russia, Romania and The Aegean coasts of Turkey (Urla sample), while Haplotype I covers the Black Sea waters of Bulgaria, Georgia, Ukraine, Turkey and the Aegean coasts of Greece.

The 6 genotypes which were not matched with any of the haplotypes were from Karaburun (5), Kefken (1), Kıyıköy (1) and Russia (1). Four of the Karaburun samples which did not match with any haplotypes were unique genotypes.

The Urla sample, which was from the Aegean coast of Turkey, completely matches one of the Zonguldak genotypes. These two samples together gave

Genotype 20 which corresponds the most common haplotype (Haplotype I) found in Viaud-Martinez *et al.*, 2007. The Haplotype I consists of 5 samples from the Aegean coast of Greece.

Table 3.2 Genotype - haplotype affiliations

Genotype	Location	# Samples	Possible Equivalent Haplotype*	Accesion No.	Location*	# Samples*
1	Karaburun Kıyıköy Ereğli Zonguldak Fatsa Şile Rumeli Feneri Romania (BS)	12 3 1 1 1 1 1 1	I	EF063646	Bulgaria (BS) Georgia (BS) Ukraine (BS) Turkey (BS) Greece (AS)	8 6 40 17 5
2	Russia (BS)	1	I	EF063646	Bulgaria (BS) Georgia (BS) Ukraine (BS) Turkey (BS) Greece (AS)	8 6 40 17 5
3	Sinop Karaburun	1 1	XXXII	EF63110	Greece (AS)	1
4	Karaburun Kefken Russia (BS)	1 1 1				
5	Karaburun	1				
6	Karaburun	1				
7	Şile	1	II	EF063647	Bulgaria (BS)	1
8	Kıyıköy	1				
9	Karaburun	1				
10	Rumeli Feneri İğneada	1 1	VIII	EF063653	Turkey (BS) Georgia (BS) Ukraine (BS)	1 2 4
11	Şile	1	XVI	EF063661	Ukraine (BS)	1
12	Karaburun	1	XVI	EF063661	Ukraine (BS)	1
13	Karaburun	1	XVI	EF063661	Ukraine (BS)	1
14	Karaburun	1				
15	Karaburun	1	X	EF063655	Turkey (BS) Ukraine (BS)	1 3
16	Karaburun	2	I	EF063646	Bulgaria (BS) Georgia (BS) Ukraine (BS) Turkey (BS) Greece (AS)	8 6 40 17 5
17	Karaburun Fatsa	2 1	I	EF063646	Bulgaria (BS) Georgia (BS) Ukraine (BS) Turkey (BS) Greece (AS)	8 6 40 17 5
18	Şile	1	I	EF063646	Bulgaria (BS) Georgia (BS) Ukraine (BS) Turkey (BS) Greece (AS)	8 6 40 17 5
19	Şile	1	I	EF063646	Bulgaria (BS) Georgia (BS) Ukraine (BS) Turkey (BS) Greece (AS)	8 6 40 17 5
20	Urla Zonguldak	1 1	I	EF063646	Bulgaria (BS) Georgia (BS) Ukraine (BS) Turkey (BS) Greece (AS)	8 6 40 17 5

* According to Viaud-Martinez et al., 2007

3.2.2 16S Results

A 532 bp fragment of the 16S rDNA gene was used for alignment from a total of 44 harbour porpoises. In addition to genotypes found in Black Sea samples, 1 more sequence, NC005280_16s, was added to analysis from Gen-Bank which is part of complete mitochondrial genome of *Phocoena phocoena*. Our sequences align with NC005280_16S from base position 879 to 1411.

Two genotypes were defined from 44 Black Sea individuals with 1 base substitution. Most common genotype (Genotype 1) was observed for 42 individuals and Genotype 2 was observed for 2 individuals. The most frequent genotype (Genotype 1) represented 95.4% of all individuals analysed while the latter genotype (Genotype 2) represented in 4.6% of the Black Sea harbour porpoises.

NC005280 sequence was completely concordant with Genotype 1. Variable sites of aligned sequences from 45 harbour porpoises and the genotype affiliations of the samples can be seen in Figure 3.21 and Table 3.3.

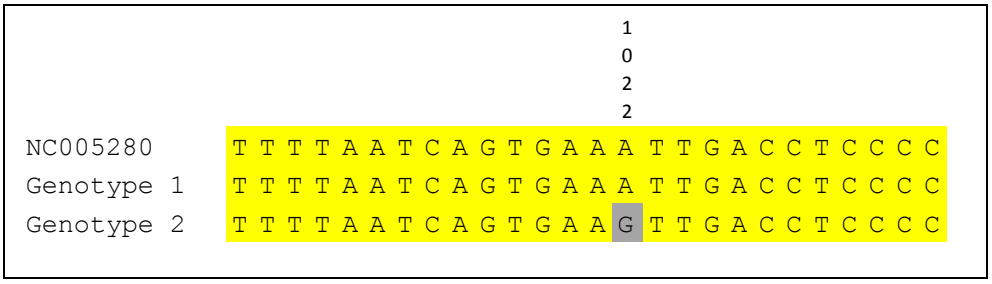

Figure 3.21 The variable sites in 2 genotypes identified for 44 Black Sea samples and 1 sample retrived from Gen-Bank. Numbered site refers to published 16S sequence for NC005280 (Arnason *et al.*2004).

Table 3.3 16S genotype affiliations across the Black Sea harbour porpoises and 1 sample from Gen-Bank

	Genotype 1	Genotype 2
Location	Karaburun (23) Kıyıköy (3) Şile (3) Fatsa (2) Russia (2) Rumeli Feneri (2) Ereğli (2) Zonguldak (2) Kefken (1) İğneada (1) Romania (1) NC005280 (1)	Kıyıköy (1) Karaburun (1)

Because only two genotypes were identified from 16S sequences, phylogenetic analyses were not done.

3.2.3 COI Results

A 549 bp fragment of the COI gene was used for alignment from a total of 44 harbour porpoises. Our fragment aligns with the published *Phocoena phocoena* complete mitochondrial genome sequence from 5532 to 6081. (NC_005280).

Eight genotypes were defined from 44 Black Sea individuals with 3 parsimony informative sites, 2 insertions and 12 base substitutions. 5 of these genotypes were shared between two or more individuals and the remaining 3 were unique. The most frequent genotype (Genotype 1) represented 70.5% of all 44 individuals analyzed while the next most frequent genotype (Genotype 2) were found in 4.7% of the Black Sea harbour porpoises.

In addition to the 8 genotypes found in Black Sea samples, 3 more genotypes were added to the analysis from Gen-Bank with accession numbers EU139290, EU139292 and NC_005280 to improve phylogenetic accuracy. The nexus block which summarises the 47 samples' corresponding COI genotypes can be seen in Figure 3.22. The aligned COI region sequences from 47 harbour porpoises showing variable sites can e seen in Figure 3.23. The genotype affiliations of all 47 samples can be seen in Table 3.4. Sequence position numbers are arranged according to COI sequence of NC_005280 which is the complete mitochondrial genome of *Phocoena phocoena.*

```
#NEXUS
begin data;
       dimensions ntax=11 nchar=14;
       format missing=? gap=-
datatype=dna;
       matrix

NC_005280 T-ACGTAAATGCCA
EU139290  T-ATATGAGTACCA
EU139292  T-ATATGAGTACTA
Genotip_1 T-ACACAAACGCCA
Genotip_2 C-ACACAAACGCCA
Genotip_3 T-ACACAAACGCCG
Genotip_4 T-ACACAAACGTCA
Genotip_5 T-GCACAAACGCCA
Genotip_6 T-ACACAGACGCCA
Genotip_7 TAACACAAACGCCA
Genotip_8 TCACACAAACGCCA
;
end;
```

Figure 3.22 Nexus block of the COI genotypes the 55 samples correspond to

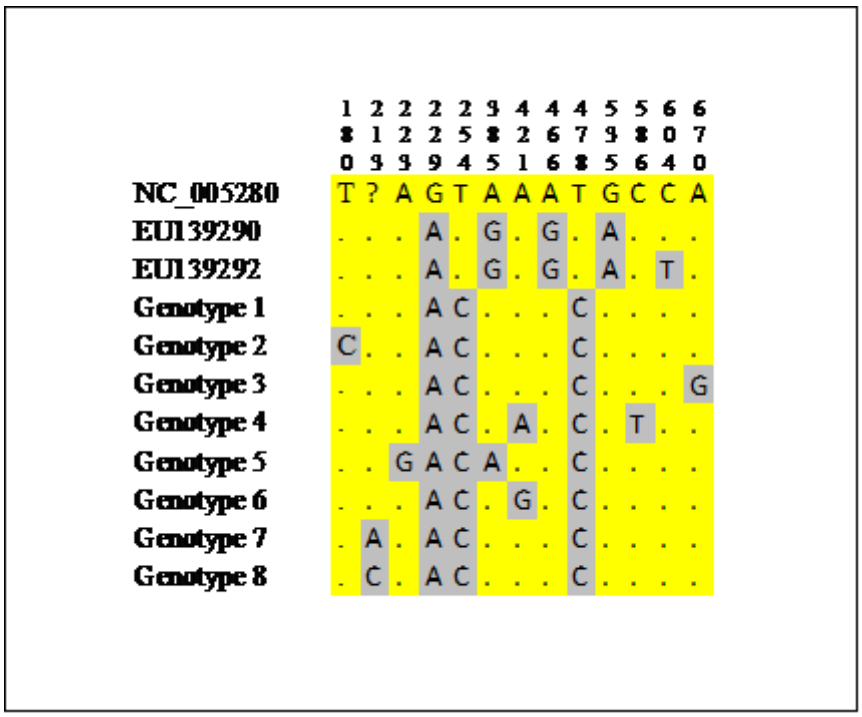

Figure 3.23 The variable sites in 11 genotypes identified for 44 Black Sea samples and 3 Atlantic samples retrieved from Gen-Bank (*dots* indicate identity with first sequence and dashes indicate insertion deletion events). Numbered sites refer to published COI sequence for *Phocoena phocoena* (Arnason *et al.*2004).

Table 3.4 COI genotype affiliations across the Black Sea harbour porpoises and 2 samples from the Atlantic used for comparison

Location of Samples	Genotype 1	Genotype 2	Genotype 3	Genotype 4	Genotype 5	Genotype 6	Genotype 7	Genotype 8	Genotype 9	Genotype 10
Karaburun/ İstanbul	15	4	1	1	0	1	1	1	0	0
Romania	1	0	0	0	0	0	0	0	0	0
Ereğli/ Zonguldak	1	0	0	0	0	0	0	0	0	0
Şile/ İstanbul	3	0	0	0	0	0	0	0	0	0
Sinop	1	0	0	0	0	0	0	0	0	0
İğneada/ Kırklareli	1	0	0	0	0	0	0	0	0	0
Zonguldak	2	0	0	0	0	0	0	0	0	0
Russia	2	0	0	0	0	0	0	0	0	0
Kefken/ Kocaeli	1	0	0	0	0	0	0	0	0	0
Kıyıköy/ Kırklareli	3	0	0	1	0	0	0	0	0	0
Fatsa/ Ordu	0	0	0	0	1	0	0	0	0	0
Rumeli Feneri/İstanbul	0	1	1	0	0	0	0	0	0	0
EU139290/ Atlantic	0	0	0	0	0	0	0	0	1	0
EU139292/ Atlantic	0	0	0	0	0	0	0	0	0	1

The phylogenetic trees obtained for COI with the two phylogenetic inference methods used (maximum parsimony and bayesian inference) both resulted in similar topologies. Thus, a single tree with maximum parsimony and posterior probability values represented on concordant nodes was chosen to present the data. The consensus tree of maximum parsimony analysis given by PAUP and bayesian inference posterior probabilities given by MrBayes are can be seen in Figure 3.24.

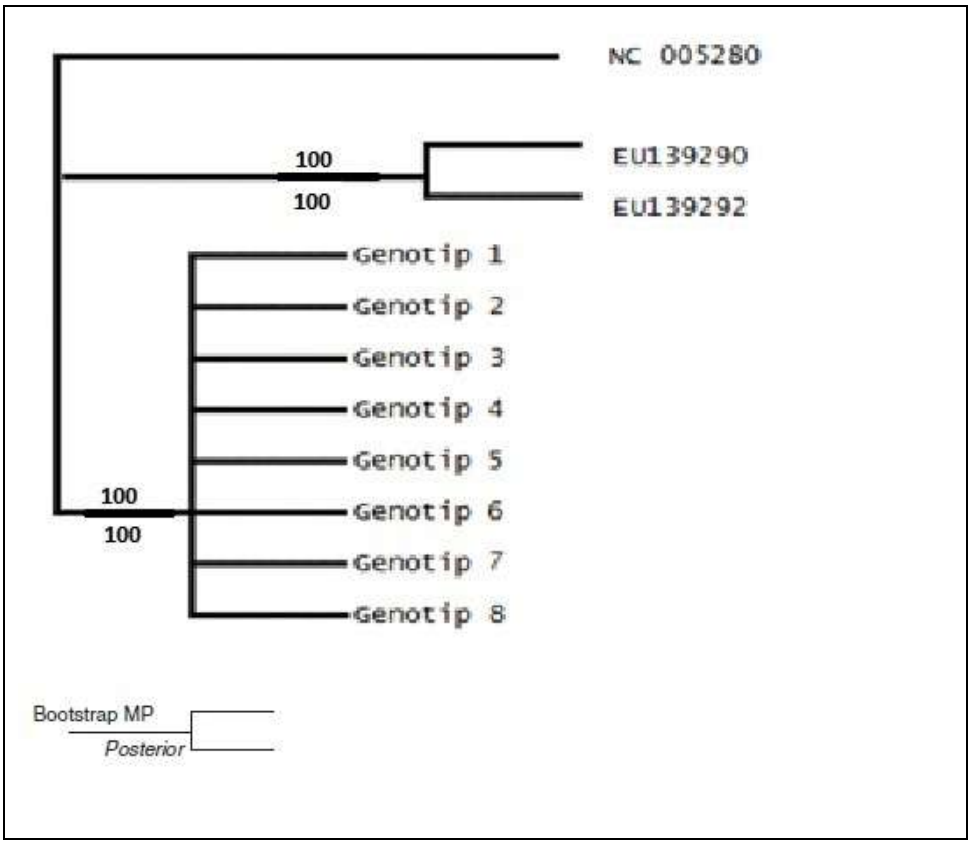

Figure 3.24 Phylogenetic tree obtained for *Phocoena phocoena* cyctochrome c oxidase I (COI) sequences. Bootstrap values for maximum parsimony (MP) and bayesian inference (BI) are above branches while posterior probability support values based on the bayesian reconstruction are shown below branches

3.2.4 Overall Results

COI, 16S and Dloop Genotypes affiliations of *P.p* samples are summarised in Table 3.5.

Table 3.5 COI, 16S and Dloop genotypes affiliations of P.p

	Sample Name	Dloop	COI	16S
1	1. Romania	1	1	1
2	2. Ereğli / Zonguldak	-	1	1
3	3. Şile / İstanbul	7	1	1
4	4. Ereğli / Zonguldak	1	-	1
5	7. Sinop	3	1	-
6	8. İğneada / Kırklareli	10	1	1
7	9. Urla / İzmir	20	-	-
8	12. Zonguldak	20	1	1
9	13. Zonguldak	1	1	1
10	14. Karaburun / İstanbul	1	1	1
11	15. Russia	4	1	1
12	16. Russia	2	1	1
13	17. Kefken / Kocaeli	4	1	1
14	18. Karaburun / İstanbul	4	1	1
15	19. Kıyıköy / Kırklareli	1	1	1
16	20. Kıyıköy / Kırklareli	1	4	2
17	21. Kıyıköy / Kırklareli	8	1	1
18	22. Kıyıköy / Kırklareli	1	1	1
19	24. Fatsa / Ordu	1	5	1
20	25. Fatsa / Ordu	17	1	1
21	27. Karaburun / İstanbul	1	1	1
22	28. Karaburun / İstanbul	5	1	1
23	29. Karaburun / İstanbul	14	1	1
24	30. Karaburun / İstanbul	6	1	1
25	31. Karaburun / İstanbul	1	6	1
26	32. Karaburun / İstanbul	1	1	1
27	33. Karaburun / İstanbul	1	1	1
28	34. Karaburun / İstanbul	13	1	1
29	35. Karaburun / İstanbul	17	1	-
30	36. Karaburun / İstanbul	12	7	1
31	37. Karaburun / İstanbul	1	1	1
32	38. Karaburun / İstanbul	16	2	1
33	39. Karaburun / İstanbul	16	2	1
34	40. Karaburun / İstanbul	1	1	1
35	41. Karaburun / İstanbul	17	1	1
36	42. Karaburun / İstanbul	1	4	2
37	43. Karaburun / İstanbul	1	2	1
38	44. Karaburun / İstanbul	9	3	1
39	45. Karaburun / İstanbul	1	-	1
40	46. Karaburun / İstanbul	1	8	1
41	47. Karaburun / İstanbul	15	2	1
42	48. Karaburun / İstanbul	3	1	1
43	49. Karaburun / İstanbul	1	1	1
44	50. Rumeli Feneri / İstanbul	10	3	1
45	51. Rumeli Feneri / İstanbul	1	2	1
46	52. Şile / İstanbul	18	1	1
47	53. Şile / İstanbul	19	1	-
48	54. Şile / İstanbul	11	-	1
49	55. Şile / İstanbul	1	-	-

Individuals which show identical genotype combinations are given in Table 3.6. 24 different genotypic combinations across each of the three segments were found in 39 samples excluding the ones which failed to give genotype from all segments.

The most frequent genotype combination had samples from Romania, Kıyıköy, Karaburun and Zonguldak. Three genotype combinations had samples from neighbouring locations and three genotype combinations had samples from geographically different locations like Russia, Kefken, Karaburun or Karaburun,Fatsa or Romania, Kıyıköy, Zonguldak, Karaburun.

Table 3.6 Different genotype combinations determined

Sample No	Location	Dloop	COI	16S
1	Romania	1	1	1
22	Kıyıköy / Kırklareli	1	1	1
13	Zonguldak	1	1	1
14	Karaburun / İstanbul	1	1	1
27	Karaburun / İstanbul	1	1	1
32	Karaburun / İstanbul	1	1	1
33	Karaburun / İstanbul	1	1	1
37	Karaburun / İstanbul	1	1	1
41	Karaburun / İstanbul	1	1	1
49	Karaburun / İstanbul	1	1	1
20	Kıyıköy / Kırklareli	1	4	2
42	Karaburun / İstanbul	1	4	2
43	Karaburun / İstanbul	1	2	1
51	Rumeli Feneri / İstanbul	1	2	1
15	Rumia	4	1	1
17	Kefken / Kocaeli	4	1	1
18	Karaburun / İstanbul	4	1	1
38	Karaburun / İstanbul	16	2	1
39	Karaburun / İstanbul	16	2	1
44	Karaburun / İstanbul	17	1	1
25	Fatsa / Ordu	17	1	1
46	Karaburun / İstanbul	9	3	1
50	Rumeli Feneri / İstanbul	10	3	1
3	Şile / İstanbul	7	1	1
8	İğneada / Kırklareli	10	1	1
12	Zonguldak	20	1	1
16	Rumia	2	1	1
21	Kıyıköy / Kırklareli	8	1	1
52	Şile / İstanbul	18	1	1
48	Karaburun / İstanbul	3	1	1
34	Karaburun / İstanbul	13	1	1
29	Karaburun / İstanbul	14	1	1
28	Karaburun / İstanbul	5	1	1
30	Karaburun / İstanbul	6	1	1
45	Karaburun / İstanbul	1	8	1
31	Karaburun / İstanbul	1	6	1
24	Fatsa / Ordu	1	5	1
36	Karaburun / İstanbul	12	7	1
47	Karaburun / İstanbul	15	2	1

3.2.5 Tursiops truncatus, Stenella coerualba and Delphinus delphis Results

Delphinus delphis sample and 1 of the *Stenella coerualba* samples failed to give PCR products. Only 3 *Tursiops truncatus* samples and 1 *Stenalla coerualba* sample were analysed phylogenetically. Dloop was investigated for 2 *T.t* samples and 1 *S.c* sample, 16S was investigated for 3 *T.t* and 1 *S.c* sample. COI was not able to be investigated for any of the samples because all samples failed to give COI PCR.

3.2.5.1 Tursiops truncatus Results

576 bp length Dloop alignment starts with the 24th bp of the published tRNA product of NC_012059 sequence. According to Dloop results Kuşadası/Aydın sample completely aligned with published AY963608 sequence which is sampled from the western Mediterranean, Lapseki/Çanakkale sample completely aligned with published AY963599 sequence which is from the eastern Mediterranean. Alignment can be seen in Figure 3.25.

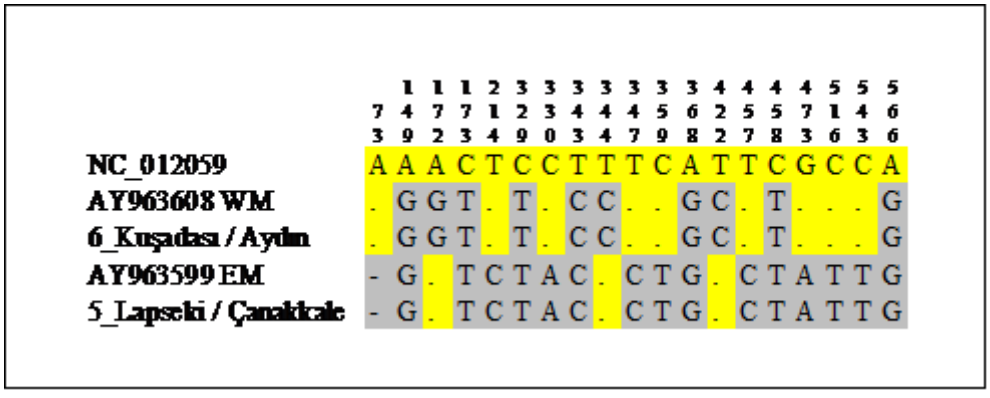

Figure 3.25 The variable sites in *T.t* Dloop alignment (*dots* indicate identity with first sequence and dashes indicate insertion deletion events)

A 538 fragment of the 16S gene was used for alignment from a total of 3 *Tursiops truncatus.* Our fragment aligns with the published *Tursiops truncatus* complete mitochondrial genome sequence starting from 870th bp (NC_012059). All 3 samples

represent different genotypes with a total of 6 base substitutions. Alignment can be seen in Figure 3.26.

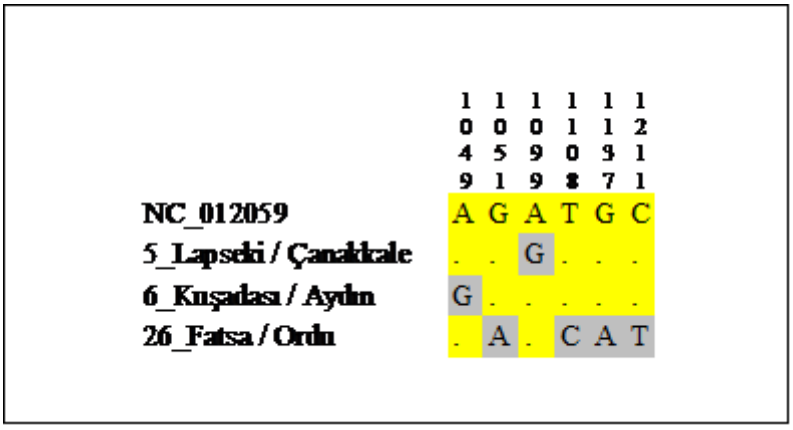

Figure 3.26 The variable sites in *T.t* 16S alignment (*dots* indicate identity with first sequence)

3.2.5.2 Stenella coerualba Results

S.c sample was aligned with *S.c* mitochondrial complete genome sequence of a Pacific Ocean sample retrieved from Gen-Bank. 535 bp length Dloop alignment starts with the 1st base of the published Dloop NC_012053 sequence. Urla/ İzmir (Aegean coasts of Turkey) sample differed from this sequence with 14 base substitutions.

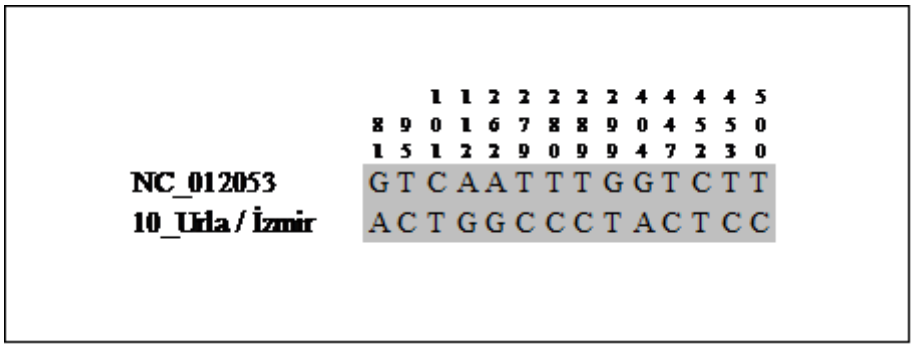

Figure 3.27 The variable sites in S.c Dloop alignment (*dots* indicate identity with first sequence).

In addition to the complete genome sequence retrieved from Gen-Bank, two 16S partial sequences were also added from Gen-Bank with accession number EU685097 and AJ010816. 529 bp length alignment starts with 937th base of the published 16S region of NC_012053 sequence. The alignment of these 5 samples differs in one base substitution in EU685097 sequence. Our sample which differed from NC_012053 sample with 14 base substitutions in Dloop, did not differed from 16S sequence EU685097.

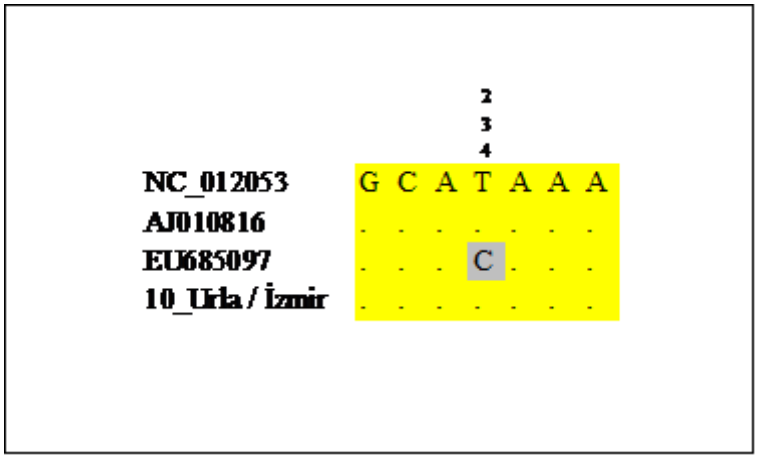

Figure 3.27 The variable sites in S.c 16S alignment (*dots* indicate identity with first sequence).

CHAPTER FOUR
DISCUSSION AND CONCLUSIONS

In this study, a total of 53 samples of small cetaceans (49 *Phocoena phocoena*, 3 *Tursiops truncatus*, and 1 *Stenella coerualba*) were investigated genetically using mtDNA markers; Dloop, 16S rDNA and COI. Also sequences retrieved from Gen-Bank were added to the alignments and phylogenetic analysis in order to improve genetic accuracy. With using mtDNA variations we sought to test the hypothesis that the Black Sea harbour porpoise population is fragmented into subpopulations.

The idea of presence of stocks / subpopulations in the Black Sea-Sea of Azov has been suggested by few scientists. Mikhalev (2009), proposed that harbour porpoises form relatively stable aggregations in the north eastern, north western, south eastern and south western Black Sea according to aerial and line transect surveys. Gol'din, suggested that there are more than 4 subpopulations: the subpopulations above listed, Marmara Sea and Sea of Azov subpopulations (Viaud-Martinez *et al.*, 2007; Gol'din, 2004a, 2004b; Gol'din pers. comm., July 2011). Also Hammond *et al.* (2008), claimed that the Black Sea harbour porpoise may consist of three or more subpopulations including those that spend much of the year in geographically and ecologically different areas, such as The Azov Sea, the north western Black Sea and the Sea of Marmara.

The Black Sea with its specific oceanographic characteristics like low salinity, seasonal fluctuations of water temperature and large amounts of anoxic waters below 100-250 m represents a unique habitat (Birkun, 2002). Resident fish stocks in the Black Sea are also might lead Black Sea harbour porpoises to show a high degree of geographic isolation in their habitat. Isolation of the Black Sea harbour porpoises has long been suggested on the basis of the absence of the species in the Mediterranean Sea (Frantzis *et al*., 2001), morphological differences (Gol'din, 2004b) and private mtDNA alleles (Rosel *et al.*, 1995). Our COI results, which had retrieved sequences from the Atlantic, supported this hypothesis with the formation of 2 distinct clades.

One clade had Atlantic samples and the other had Black Sea samples gathered in this study.

The samples collected for this study were mainly from the western Black Sea coast of Turkey, where the turbot fishing is more intense. A variety of samples which belongs to the other Black Sea countries were retrieved from Gen-Bank and added to the analysis. This addition made the samples more distributed and increased the chance of identifying subpopulation structures in different parts of the Black Sea. Although we found statistically significantly different phylogenetic groups we did not find distinct population structures in the Black Sea. Similar studies had concluded likewise although they were focussed on comparison between the Black Sea populations and the Atlantic or the Pacific populations instead of examining variability within the Black Sea (Fontaine *et al*., 2007; Fontaine *et al*., 2010; Rosel *et al.*, 1995; Tolley & Rosel, 2006; Viaud-Martinez *et al.*, 2007).

4.1 Dloop

As expected, the most of the genotypic differences were found in the Dloop sequence which is the most variable part of mtDNA (Avise *et al.*, 1987). Within this study, totally 20 genotypes were identified in the Black Sea with 13 unique genotypes. Among them the genotype which has the highest frequency of occurrence, represents 43% of the samples collected from Karaburun, Kıyıköy, Rumeli Feneri, Şile, Ereğli, Zonguldak, Fatsa and Romania. The remarkable broadness of the distribution range may indicate the long distance mobility of the species. This discontinuity of the most common genotype in the rest of the sampling areas could be caused by low sampling size of these locations. Among 20 genotypes, there are 13 unique genotypes and 7 of these 13 unique genotypes are from Karaburun, where the largest number sample size, 25 specimens was obtained. Thus, the highness in number of unique genotypes seems to be depended to sample size.

This 7 different and unique genotypes show 7 different maternal lineages in Karaburun and this might be indicate the region as a frequently used zone for harbour porpoise population in the Black Sea.

Considering the 2 phylogenetic methods (ML and MP), there is only one clade formation. 3 of the formed branches in maximum likelihood consensus tree with 100% probability are not supported by maximum parsimony. The clade supported by both of the phylogenetic methods displays 2 genotypes (Genotype 9 & 10) from geographically neighbouring locations, i.e., Karaburun, Rumeli Feneri and İğneada. This clade presumably represents a distinctive character in the subpopulation structure in the western Black Sea coast of Turkey. Such a distinction requires a consistent homogeneity in gene exchange during several generations within a group consisted by certain individuals living together. However, there is no sufficiently representative information on the population dynamics of this species distributed in Black Sea

But even though this clade was supported by 2 methods, other samples which were collected from the same locations, showed different genotypes and these genotypes were all in different branches or in different clades in the ML consensus tree. The difference in sample size can restrict the interpretation.

The Black Sea haplotypes from Gen-Bank were dispersed among our genotypes as expected. The only clade formation found by bayesian consensus tree with 100% probability, were for the samples from the Atlantic and Gibraltar, which is already considered a separate population. Other clades given by the bayesian consensus tree with less than 100% probability consisted of samples from different parts of the Black Sea. ML consensus tree gives 11 clade formations with 100% probability, only one of these clades is supported by MP consensus tree with 56% probability. This clade which was supported by ML and MP had samples from Şile, Karaburun and Bulgaria. This result also supports the distinctive character in population structure in the south western Black Sea.

In this study we analysed the first confirmed harbour porpoise sample from the Turkish Aegean Sea with the sample name Urla (Güçlüsoy, 2008). The harbour porpoise is extremely rare in the Mediterranean basin in, both historical and contemporant records (Rosel *et al.,* 2003). Frantzis (2001), concludes that only five observations of harbour porpoises are reliable indicators of the recent presence of this species in the Mediterranean Sea. Therefore, the Urla sample has great importance as it contributes to the knowledge of the species in the Aegean Sea. Nevertheless, the scarcity of observations is clear evidence that their presence in Aegean Sea is not permanent.

Six samples collected from the Greek Aegean coast corresponding two haplotypes. One haplotype (Haplotype I) corresponded to genotype of Urla sample (Genotype 20) while second haplotype (Haplotype XXXII) corresponded to different genotypes found in this study, because of the length differences between sequences. None of the consensus trees gave branch formations with Haplotype I and Genotype 20, which are corresponded sequences of Greek and Turkish Aegean samples.This suggests the Aegean sample genotype we found in this study differs from other Aegean samples according to ML, MP and BI phylogenetic inferences.

4.2 16S

Only two genotypes were identified from the collected samples. Therefore the genotypes could not be subjected to phylogenetic analysis. This level of low variability in 16S region of mtDNA was not expected. Because of this low variability, the region could be used for species identification together with Dloop as Dloop itself a variable part and 16S itself as a conservative part of mtDNA.

4.3 COI

Eight COI genotypes were found in this study except the two Atlantic sequences retrieved from Gen-Bank. Maximum parsimony and bayesian inference methods, both gave the same consensus tree with forming two different clades, one with the Atlantic samples and the second with the genotypes found in this study. These trees support the isolation of the Black Sea harbour porpoise from the Atlantic porpoises. According to the COI results, the Black Sea population is not significantly different within itself which means that it was not possible to fragment the *Phocoena phocoena* population in the Black Sea.

4.4 Overall

The most frequent genotype combination observed had samples from Karaburun, Kıyıköy, Romania and Zonguldak. Karaburun, Kıyıköy and Romania are relatively closer locations which could be the area where a possible subpopulation structure exists while Zonguldak remains relatively far. Besides, Zonguldak sample was not included to clade formations with Karaburun, Kıyıköy and Romania samples in phylogenetic trees. Considering the phylogenetic trees given by MP and ML İğneada, Karaburun and Rumeli Feneri samples formed a clade which can be interpreted as a subpopulation structure.

As mentioned before, the branch formation found for the European continental part of western Black Sea covering sampling areas Rumeli Feneri, Karaburun, Kıyıköy, İğneada, Bulgaria and Romania, could be biased due to the larger sample size in these locations.

According to Gaskin (1984) the apparent coastal nature of the species during the summer months may restrict long distance movement and this may lead a genetic differentiation among local populations. But when the swimming speed of harbour porpoises is taken into consideration the distance between sampling areas remains sufficiently close to be in their range. According to Otani *et al.* (2001), the minimum

cost of transport during underwater swimming in the harbour porpoise was 2.39–2.43 J/kg per m at an average swim speed of 1.3–1.5 m/s. However, porpoises usually swam slower to conserve energy and dive aerobically. But given the harbour porpoises swimming speed varies between 0.5 to 4.2 m/s, the 140 km distance between sampling areas İğneada and Rumeli Feneri, remains well within their range. This may explain how the same genotypes can be found far apart.

The idea of the exclusive inshore occurrence of harbour porpoises was rejected by Mikhalev (2009). According to Mikhalev (2009), being able to forage on pelagic fishes makes them regularly observed in offshore Black Sea waters, including deep areas. This may account for how Russia, Sinop and Karaburun samples can be found in same clade in the ML consensus tree.

According to Birkun & Frantzis (2008), it is known that Black Sea harbour porpoises undertake annual migrations leaving the Azov Sea and the north western Black Sea through the southern Black Sea in winter season. The south eastern Black Sea is considered as wintering area for the Black Sea harbour porpoises as well as it is wintering area for Black Sea anchovy population which is a principal prey species for harbour porpoises in cold season (Kleinenberg, 1956). Spawning ground of the Black Sea anchovy, *Engraulis encrasicolus*, can be seen in Figure 4.1.

Figure 4.1 Spawning ground of Black Sea anchovy population, *Engraulis encrasicolus,* (source UNEP Grid Arendal)

This possible subpopulation formation could be considered as a potential separate management unit. However, more genetic studies are needed to validate this subpopulation formation using larger sample sizes more evenly distributed across different locations.

4.5 *Tursiops truncatus*

Two samples were analysed for Dloop and 16S regions. According to Dloop alignments, the Lapseki / Çanakkale sample identically matched with the eastern Mediterranean sample retrieved from Gen-Bank. This was expected because Çanakkale is in the eastern Mediteranean basin too. But unexpectedly the Kuşadası / Aydın sample identically matched with the western Mediterranean sample. This could be explained by the bottlenose dolphin having come from the eastern Mediterranean. Alternatively, it may have escaped or otherwise been released from a dolphinarium in the Aegean coast of Turkey.

4.6 *Stenella coerualba*

The striped dolphin sample from Urla / İzmir was analysed for Dloop and 16S regions. While the Dloop sequence of Urla differs from the complete genome sequence of Pacific Ocean sample by 14 base substitutions; the 16S sequence identically matched the complete genome 16S sequence. These results fall in the expected known nature of these two fragments of mtDNA.

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APPENDICES

Appendix 1. Phylogenetic trees of mtDNA Dloop region of P.p. (Numbers on the branches gives the probability of each partition or clade in the tree).

Figure 1. Maximum likelihood consensus tree given by PAUP for Dloop genotypes of collected samples

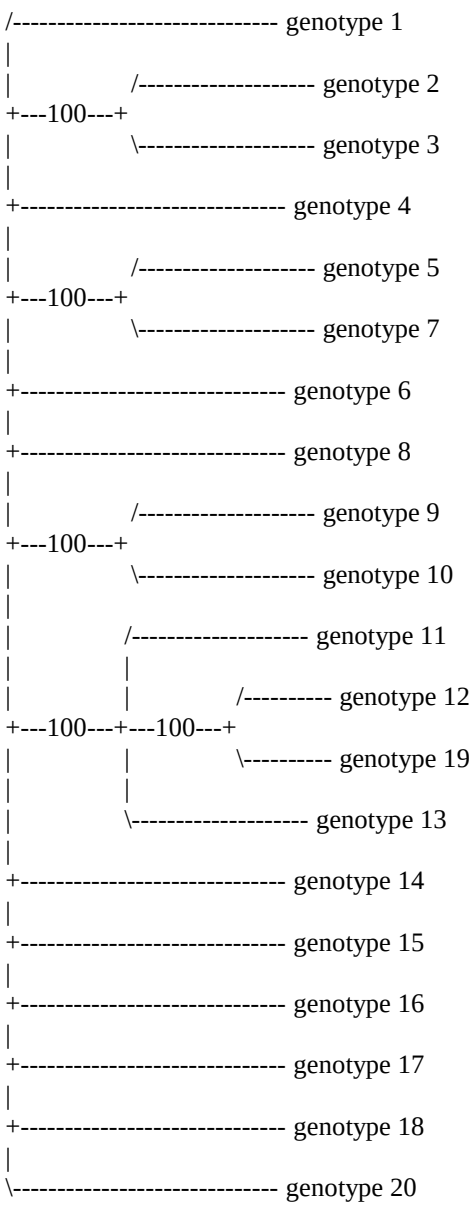


Figure 2. Maximum parsimony consensus tree given by PAUP for Dloop genotypes of collected samples

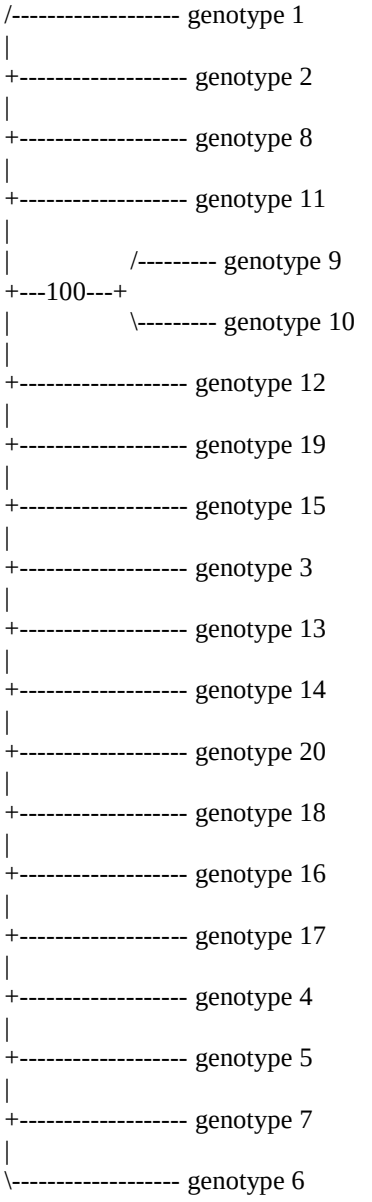
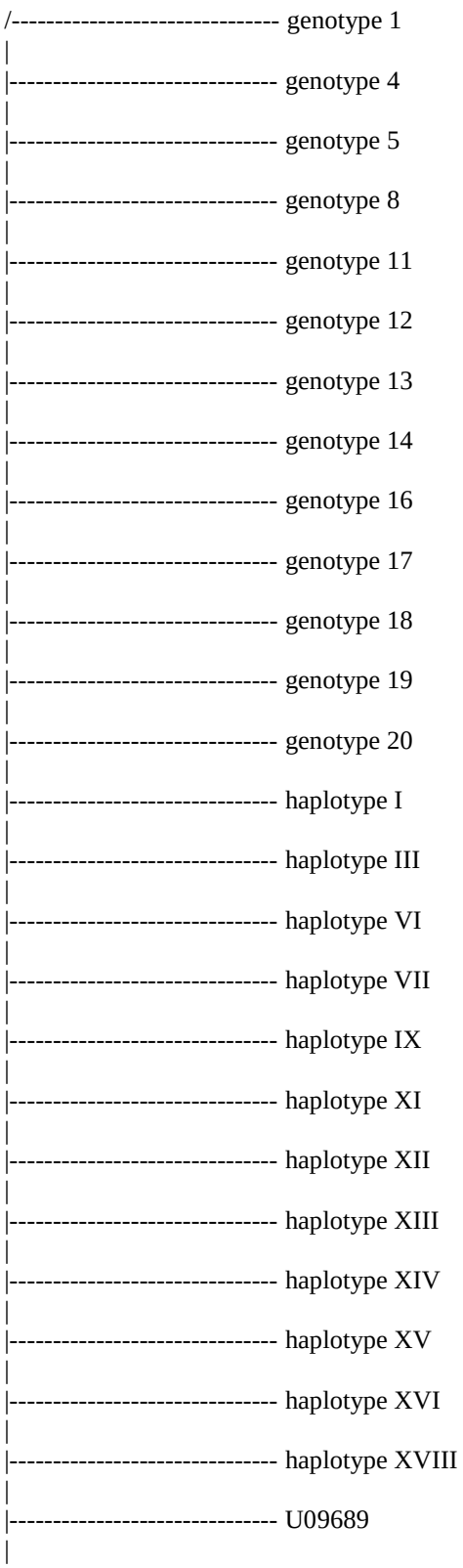


Figure 3. Bayesian consensus tree given by MrBayes for genotypes determined in this study and haplotypes retrieved from Gen-Bank



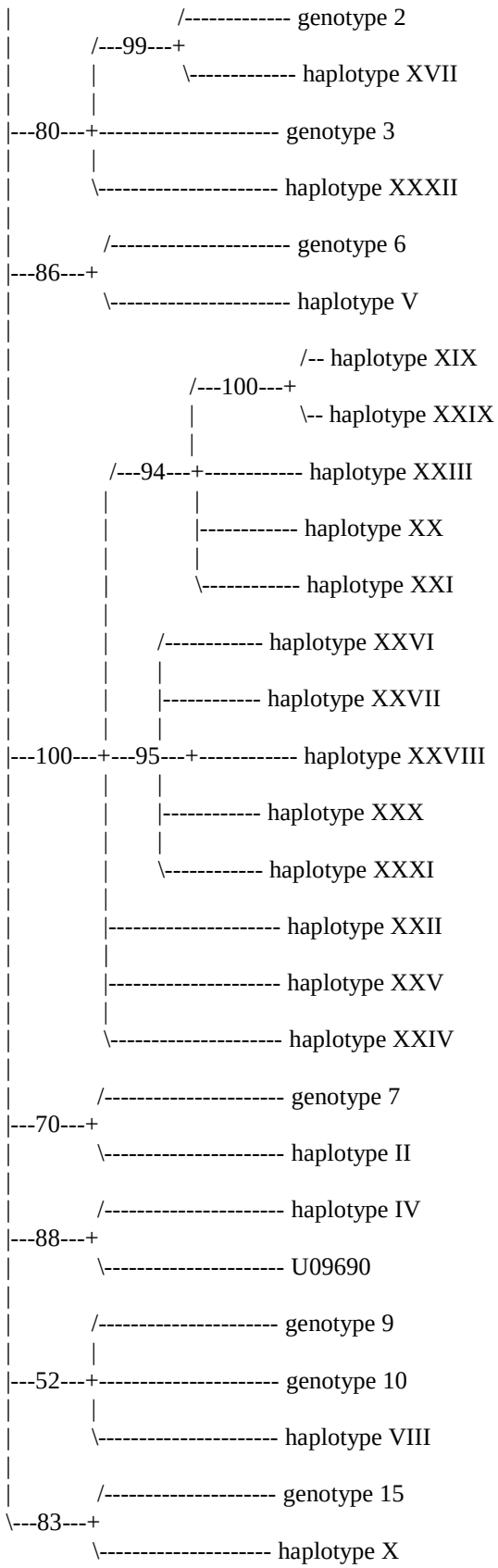
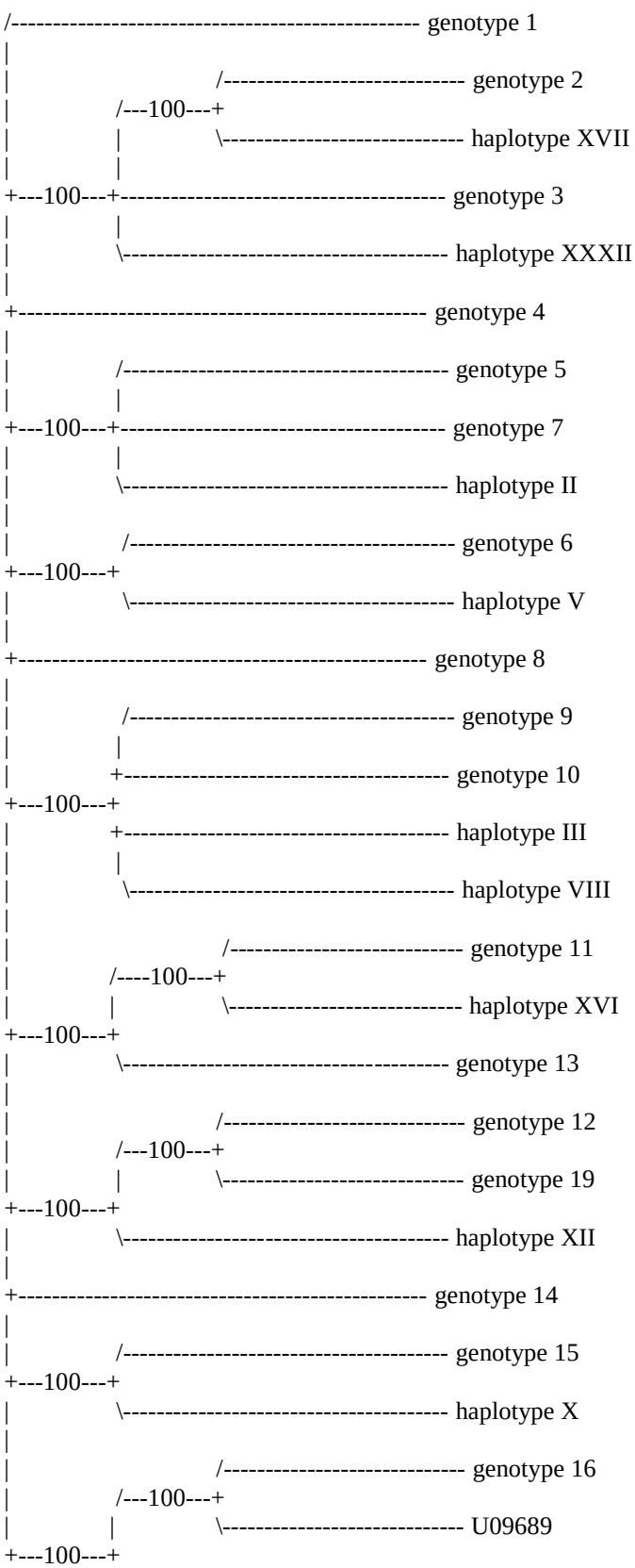

genotype 2
haplotype XVII
genotype 3
haplotype XXXII
genotype 6
haplotype V
haplotype XIX
haplotype XXIX
haplotype XXIII
haplotype XX
haplotype XXI
haplotype XXVI
haplotype XXVII
haplotype XXVIII
haplotype XXX
haplotype XXXI
haplotype XXII
haplotype XXV
haplotype XXIV
genotype 7
haplotype II
haplotype IV
U09690
genotype 9
genotype 10
haplotype VIII
genotype 15
haplotype X

Figure 4. Maximum likelihood consensus tree given by PAUP for all Dloop sequences



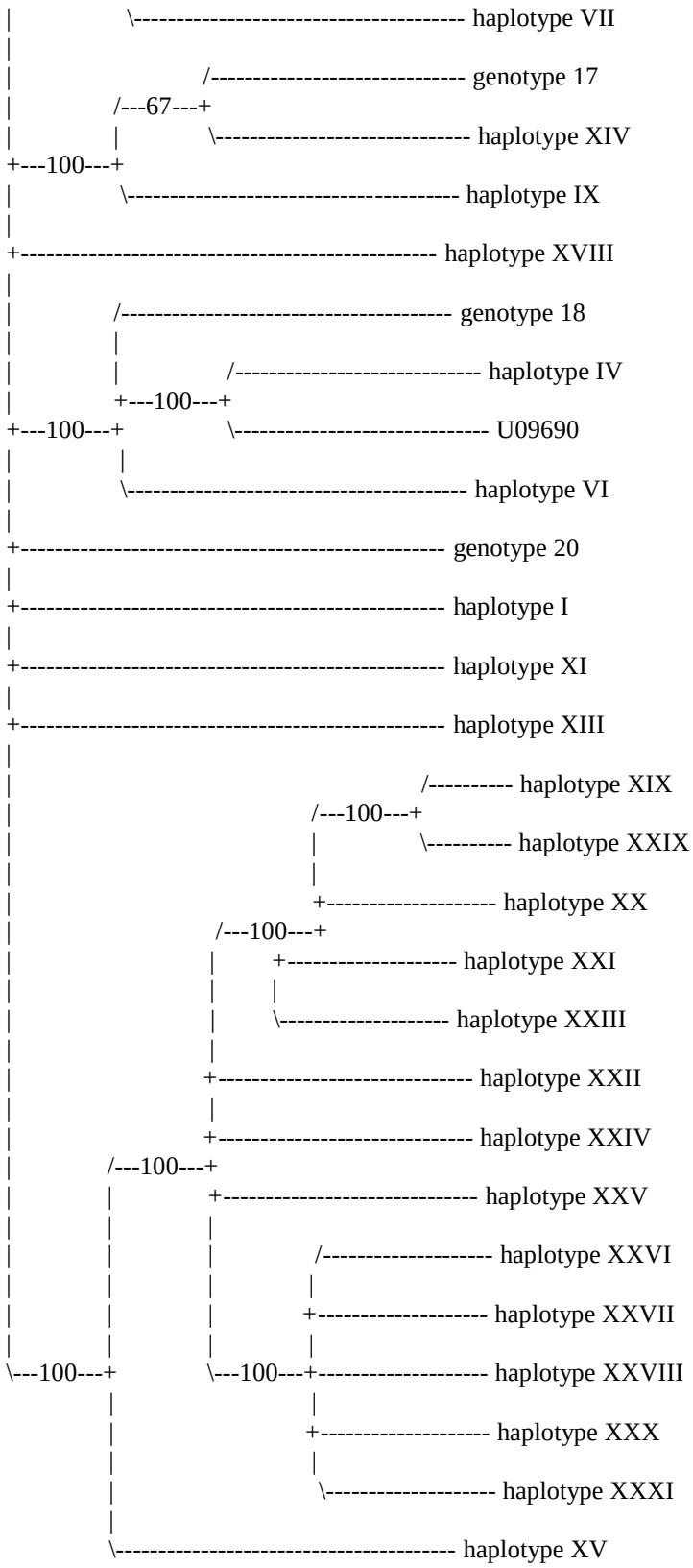
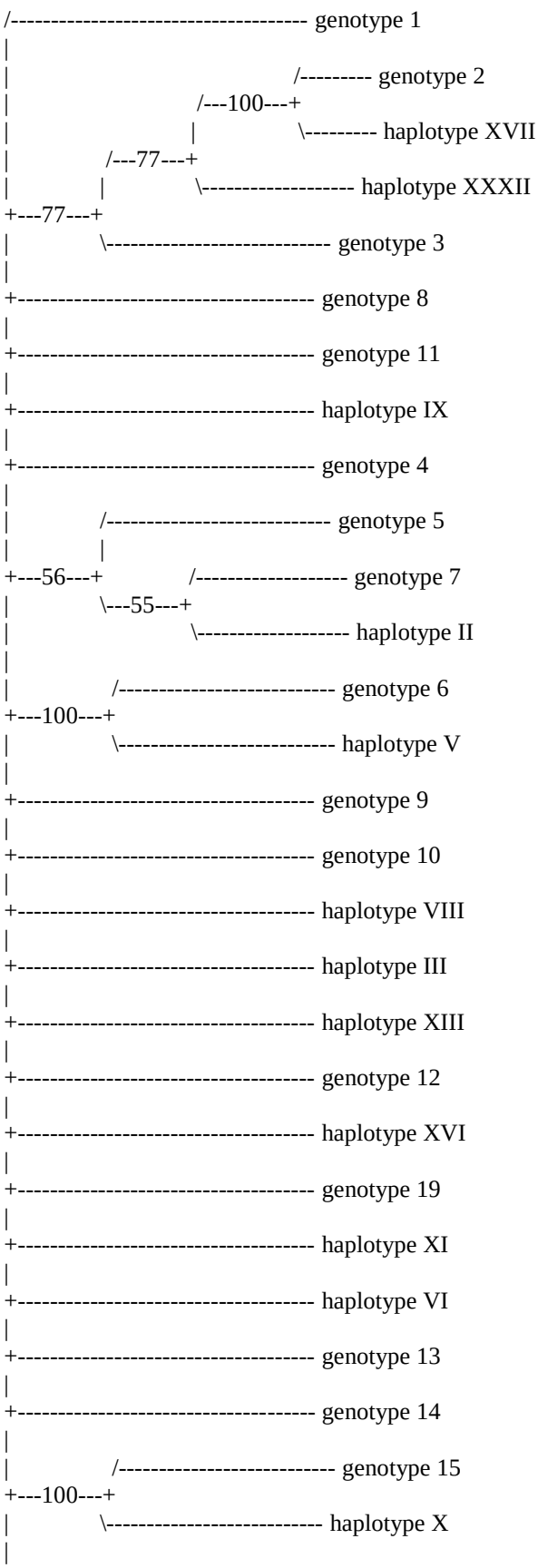
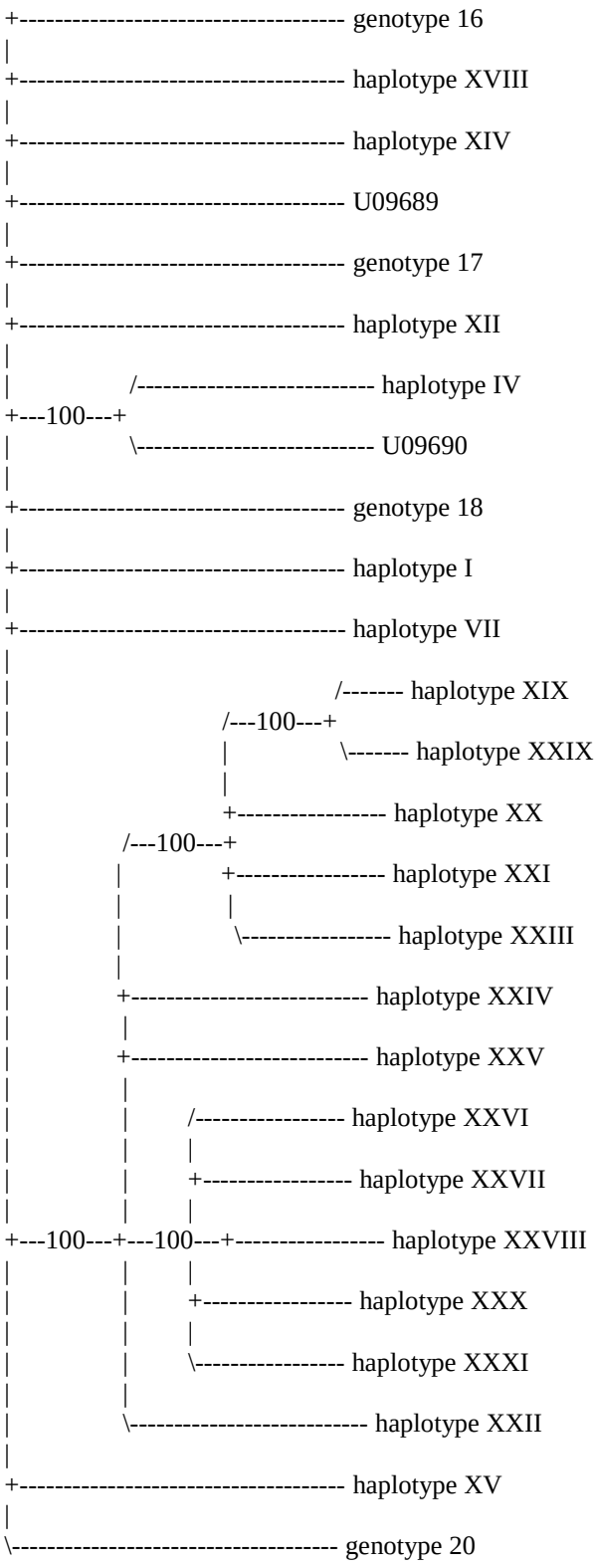

haplotype VII
genotype 17
67
haplotype XIV
100
haplotype IX
haplotype XVIII
genotype 18
haplotype IV
100
U09690
100
haplotype VI
genotype 20
haplotype I
haplotype XI
haplotype XIII
haplotype XIX
100
haplotype XXIX
haplotype XX
100
haplotype XXI
haplotype XXIII
haplotype XXII
haplotype XXIV
100
haplotype XXV
haplotype XXVI
haplotype XXVII
100
haplotype XXVIII
haplotype XXX
haplotype XXXI
haplotype XV

Figure 5. Maximum parsimony consensus tree given by PAUP for all Dloop sequences




genotype 16
haplotype XVIII
haplotype XIV
U09689
genotype 17
haplotype XII
haplotype IV
100
U09690
genotype 18
haplotype I
haplotype VII
haplotype XIX
100
haplotype XXIX
haplotype XX
100
haplotype XXI
haplotype XXIII
haplotype XXIV
haplotype XXV
haplotype XXVI
haplotype XXVII
100
100
haplotype XXVIII
haplotype XXX
haplotype XXXI
haplotype XXII
haplotype XV
genotype 20

Annexes

Annex 1

Genetically Different Small Cetaceans in Turkish Waters

1- Harbour Porpoise

The harbour porpoise namely *Phocoena phocoena* Linnaeus 1758, is distributed throughout the cold temperate and sub-polar continental shelf waters of the northern hemisphere. This is a predominantly neritic cetacean, frequenting coastal areas, shallow bays, inlets and estuaries. Populations of this species are geographically isolated. Both morphological and genetic data suggest that globally, the harbour porpoise is comprised of at least three genetically isolated populations worldwide: *P. p. phocoena* in The North Atlantic, *P. p. vomerina* in The eastern North Pacific and *P. p. relicta* in Black Sea/Sea of Azov (Chiviers *et al.*, 2002; Fontaine *et al*., 2007; Fontaine *et al*., 2010; Rosel *et al.*, 1995; Rosel *et al.*, 1999a; Rosel, Tiedemann & Walton, 1999b; Tolley & Rosel, 2006; Viaud-Martinez *et al.*, 2007).

Figure 1.1 Worldwide distribution of the three subspecies of harbour porpoises (IUCN)

Morphological studies focused on whether Black Sea harbour porpoises are unique have led to opposite conclusions. For example, Zalkin (1938) considered the *Phocoena phocoena relicta* Abel, 1905 as a subspecies based on morphological comparisons while Barabash-Nikiforov (1940) considered this population to be a

new species. Tomilin (1957) noted that the Black Sea form differs from porpoises from the North Atlantic and North Pacific in having an average lesser height of the occipital condyles and narrower condylar width, as well as absence of tubercles on the front margins of the dorsal fin and flippers. Most recently, Gol'din (2004b) supported a subspecies classification for the Black Sea – Sea of Azov harbour porpoise, based on his own body size and skull measurements of Azov porpoises and a review of preview studies. Viaud-Martínez *et al.* (2007) compared 45 specimens from The Black Sea with 132 from The North Atlantic and concluded that The Black Sea form on average is smaller, has a smaller skull, wider and long rostrum, lesser orbital length, smaller internal nares, lesser condylar width, and a larger occipital ridge than porpoises from the North Atlantic. Viaud-Martínez *et al.* (2007), also sequenced the first 364 base pairs of the mtDNA control region for 93 porpoises from the Black Sea and 49 from the Sea of Marmara, Aegean Sea and North Atlantic and found no shared haplotypes and a strong differentiation between the Black Sea and the North Atlantic, suggesting their separation for thousands of years.

The Black Sea population is completely isolated from the nearest *P. phocoena* population in the north eastern Atlantic by a wide range discontinuity in the Mediterranean Sea, from the Northern Aegean Sea to the Strait of Gibraltar (Frantzis *et al.,* 2001; Viaud-Martinez *et al.,* 2007) although there is no agreement on when it happened (Frantzis *et al*., 2001; Rosel *et al.,* 1995).

P. phocoena relicta's range includes the Black Sea and adjacent water bodies such as the Azov Sea, the Kerch Strait (Zalkin, 1938), the Marmara Sea, the Bosphorus Strait (Öztürk & Öztürk, 1997), the Dardanelles Straits (Birkun & Frantzis, 2008; Guclusoy, 2006) connecting the Marmara and the northern Aegean Seas and also the northern Aegean Sea (Frantzis *et al*., 2001).

Genetic studies done on the North Pacific and North Atlantic subspecies suggested the harbour porpoise populations are subdivided into relatively small demographically isolated subpopulations (Chivers et al., 2002; Hammond *et al.*, 2002; Tolley *et al.*, 2001; Rosel *et al.*, 1999a, 1999b; Tolley & Rosel, 2006; Rosel *et al.*, 1995).

It is not known whether the Black Sea harbour porpoise population consists of more than one distinct subpopulation. Mikhalev (2009), proposed 4 subpopulations according to aerial and line transect surveys. Gol'din (2004a, 2004b), suggested the presence of more than 4 subpopulations. And also according to Hammond *et al.* (2008), the population of *P. p. relicta* may consist of three or more subpopulations including those that spend much of the year in geographically and ecologically different areas, e.g. the Azov Sea, north western the Black Sea and the Sea of Marmara.

Line transect surveys have been conducted recently to estimate the Black Sea harbour porpoise abundance in different parts of the range. Results of those surveys suggest that present total population size is at least several thousand and possibly in the low tens of thousands. But there are no current estimates of total population size (Birkun & Frantzis, 2008). Nevertheless it is known that in the 20th century, the number of Black Sea harbour porpoises was dramatically reduced through massive killing for a variety of industrial uses which continued until 1983 (Buckland *et al.*, 1992).

The Black Sea Harbour Porpoise is classified as Endangered (EN) based on the criteria A1d + A4cde in the IUCN Red List of Threatened Species (Birkun & Frantzis, 2008) and in resolution 3.19 of ACCOBAMS MoP3 (ACCOBAMS report, 2007). The grounds for justification are mostly based on direct killing and high incidental mortality rates of the species.

1.2.2 Common Bottlenose Dolphin

The Black Sea common bottlenose dolphin, namely *Tursiops truncatus ponticus* Barabash-Nikiforov 1940, is differentiated genetically from other bottlenose dolphin populations. The range of the Black Sea bottlenose dolphins includes the Black Sea, Strait of Kerch, Azov Sea, and the Turkish Straits including the the Bosphorus and the Dardanelles Straits, and the Sea of Marmara, whereas the common bottlenose dolphin is widely distributed in temperate oceans and semi-enclosed seas including the Mediterranean Sea.

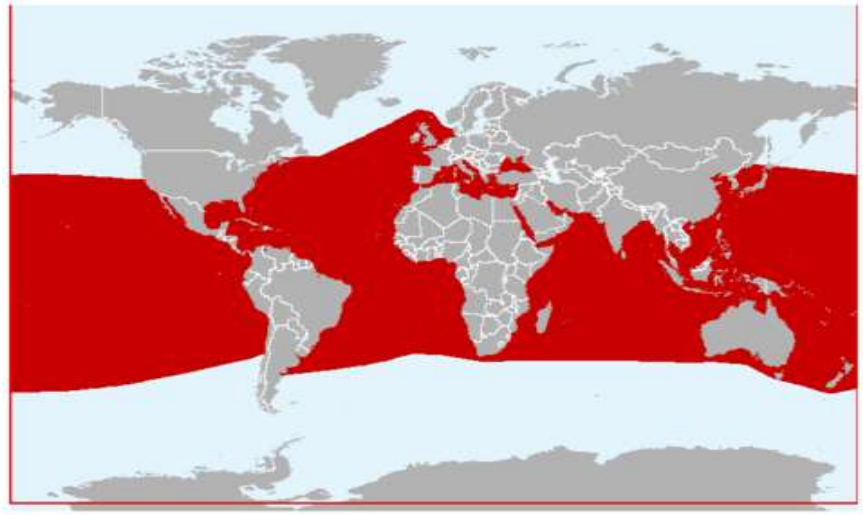


Figure 1.2 The worldwide distribution of the common bottlenose dolphin

Barabash-Nikiforov (1940) and Tomilin (1957) state that, the Black Sea specimens had on average a smaller body and skull. Also analysis of the mtDNA control region shows that the Black Sea common bottlenose dolphin population is distinctly differentiated genetically from other bottlenose dolphin populations in the eastern and western Mediterranean and the north eastern Atlantic (Natoli *et al*., 2005; Viaud-Martinez *et al*., 2008). According to Viaud-Martinez (2008), haplotype diversity and nucleotide diversity were similar in the Atlantic, Tyrrhenian and Adriatic populations but lower in the Black Sea and Aegean populations. Only six haplotypes were found in 43 northern Black Sea individuals, and only three of these were restricted to the Black Sea. Overall the Black Sea population of common

bottlenose dolphin has been accepted as isolated since its colonization. Morphologic and genetic evidence support the recognition of a valid subspecies, *Tursiops truncatus ponticus* (Birkun & Frantzis, 2008; Reeves & Notarbartolo di Sciara, 2006). The species is listed as endangered based on criterion A2cde in The IUCN Red List of Threatened Species.

1.2.3 Short Beaked Common Dolphin

The Black Sea common dolphin, namely *Delphinus delphis ponticus* (Barabash-Nikiforov, 1935), is considered as a separate subspecies. However, the justification was later criticized as not being diagnostic (Kleinenberg, 1956). Barabash-Nikiforov (1940) described the subspecies based on the external measurements of more than 20,000 dolphins. The dolphins were on average smaller, by about 20-30cm, than short-beaked common dolphins in adjacent waters.

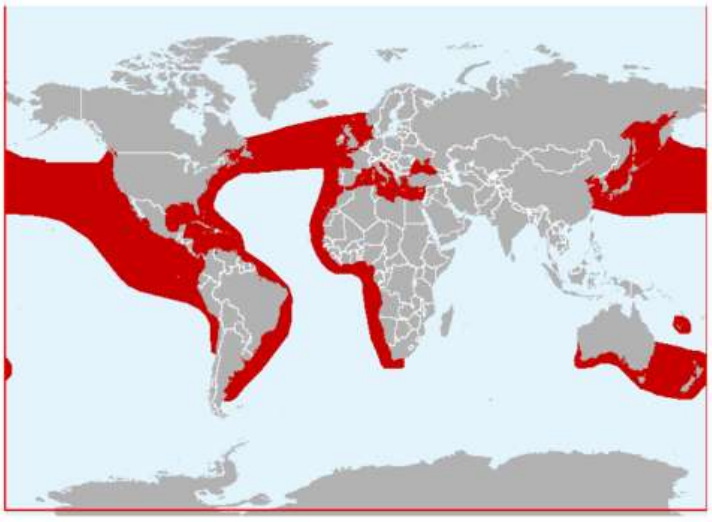


Figure 1.1 Worldwide distribution of the short beaked common dolphin

Genetic analysis suggested that differences exist between the Black Sea and the Mediterranean common dolphins. Preliminary results of a minimum spanning network based on both 428 bp of the mtDNA control region and the analysis of nine microsatellites propose the isolation of the Black Sea population from the

Mediterranean populations despite the small sample size (Natoli, 2008). Even it is not definitive at present, gene flow between the Black and Mediterranean Seas seems to be rare. Recognition of the Black Sea subspecies was also recommended by Perrin *et al.*, 2010 and the subspecies is provisionally recognized as vulnerable based on criterion A2cde in The IUCN Red List of Threatened Species.

Mediterranean subpopulation of *Delphinus delphis* is considered as distinct subpopulation according to limited gene flow from Atlantic. Genetic exchange between common dolphins from the Mediterranean Sea and Atlantic Ocean appears to involve only animals from west Mediterranean. At the eastern end of the Mediterranean, based on results obtained by Natoli (2008), the gene flow between Mediterranean and Black Sea common dolphins appears to be rare to non-existent. Once the species was commonest species in the Mediterranean Sea, the Common Dolphin has experienced a generalized and major decrease in this region during the last 30-40 years (Bearzi *et al.*, 2003). The Mediterranean subpopulation of the species recognized as endangered based on criterion A2abc in The IUCN Red List of Threatened Species.

Annex 2

Definition of Concepts Used in This Study

Population

Wells & Richmond (1995), identified more than 20 terms to describe groups generally referring to populations. According to Wells & Richmond, a population should be defined by discontinuity or disjunction in one of the following characteristics:

1. Spatial structure
2. Genetic structure
3. Demographic structure

Spatial disjunction occurs when individuals from one group cannot interact with individuals from another group. This could be caused by great distances or a physical barrier that prevents interaction. This kind of disjunction is described as the easiest to detect by Wells & Richmond (1995).

A genetic disjunction occurs when all individuals of a group share an identical set of genetic attributes that are not shared with individuals of another group. This definition of a group of individuals defined by a genetic disjunction is essentially the same as that of a phylogenetic species, as defined by Nixon & Wheeler (1990).

Demographic disjunction is formed when too few immigrants from one spatially disjunct group come into another to affect the growth rate of the group receiving the immigrants.

Subspecies

For many researchers, the concept of a subspecies is related to a geographical distribution of diversity within a species. However it is still a very controversial concept and not accepted by some authors.

Mayer (1982), points out that the concept of a subspecies contributes to a better understanding of the geographic variation of species taxa in nature. He evaluates the concept of a subspecies in the following manner:

The majority of authors, right to the end of the 19th century, defined even the subspecies essentialistically as a constant, well-defined entity at a lower level than the species. Any distinct natural population that was not considered sufficiently different to be called a separate species was called a subspecies…. After 1859 the subspecies acquired a dual biological meaning. On the one hand, it was considered the "incipient species" of Darwin, that is, as a stage in the speciation process. On the other, it was considered by certain authors like Gloger, Bergmann, and J. A. Allen to be evidence of the adaptive response of species to local climatic conditions. That the first of these two meanings was ordinarily true only for isolates while the second was particularly conspicuous for widespread continental species was not at first recognized and subsequently caused a good deal of confusion (Mayer, 1982, p593).

Subpopulation

The concept of a subpopulation is widely accepted by a variety of writers. Wells & Richmond (1995) define it as: “A group of individuals within an investigator-delimited area which have smaller range than the geographic range of the species and generally form within a population.” When a set of individuals is not spatially disjunct from other individuals within the population, the terms such as *group*, *subpopulation* or *local population* are used.

Management Units and Evolutionary Significant Units

The definition of conservation units for a species is fundamental to conduct management. Moritz (1995), suggests two distinct conservation units: Management Units (MUs) and Evolutionary Significant Units (ESUs). Management Units represent sets of populations that are currently demographically independent while Evolutionary Significant Units represent historically isolated sets of populations that together encompass the evolutionary diversity of a taxon. Both units are defined by differences in allele frequency and phylogeny.