



Shaping species conservation strategies using mtDNA analysis: The case of the elusive Mediterranean monk seal (*Monachus monachus*)



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ABSTRACT

Halting biodiversity loss is one of the major conservation challenges of our time and science-based conservation actions are required to safeguard the survival of endangered species. However the establishment of effective conservation strategies may be hampered by inherent difficulties of studying elusive animals. We used analysis of control region sequences to obtain baseline information on the genetic diversity and population structure and history of the elusive and critically endangered Mediterranean monk seal that will help define an effective conservation strategy for the species. We analyzed 165 samples collected throughout the entire extant range of the species and identified 5 haplotypes. Based on levels of genetic diversity (haplotypic diversity: 0.03; variable sites: 0.6%) the Mediterranean monk seal appears to be one of the most genetically depauperate mammals on Earth. We identified three genetically distinct monk seal subpopulations: one in the north Atlantic [Cabo Blanco vs. Aegean Sea ($F_{ST} = 0.733$; $P = 0.000$); Cabo Blanco vs. Ionian Sea ($F_{ST} = 0.925$; $P = 0.000$)] and two in the Mediterranean, one in the Ionian and another one in the Aegean Sea (Ionian vs. Aegean Sea $F_{ST} = 0.577$; $P = 0.000$). Results indicate a recent divergence and short evolutionary history of the extant Mediterranean monk seal subpopulations. Based on the results we recommend continuation of the monitoring efforts for the species and systematic collection of genetic samples and storage in dedicated sample banks. On a management level we argue that, based on genetic evidence, it is justified to manage the Atlantic and Mediterranean monk seal subpopulations as two separate management units. In Greece, the existence of two subpopulations should guide efforts for the establishment of a network of protected areas and identify the monitoring of habitat availability and suitability as an important conservation priority.

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1. Introduction

Despite recent conservation successes, biodiversity loss continues to accelerate, threatening not only the future of numerous species (Hoffmann et al., 2010), but also the ecological services provided by entire ecosystems (Worm et al., 2006). Currently more than one quarter of the world's mammalian species, usually large-bodied animals, are threatened with extinction (Anonymous, 2010), a fact that is considered a significant threat to biodiversity (Galetti and Dirzo, 2013). In this

context, science-based management and conservation actions are urgently required to safeguard the survival of these species (Boersma et al., 2001). In the case of endangered marine mammals, species that are often charismatic and capable of attracting attention and resources to conservation, the establishment of science-based, effective conservation strategies is often hampered by the inherent difficulties (i.e., high mobility, large home ranges) related to their biology.

The Mediterranean monk seal (*Monachus monachus*) is the sole extant representative of the genus *Monachus* (Scheel et al., 2014) and one of the most critically endangered marine mammals on Earth (Anonymous, 2010). Once widely and continuously distributed throughout the Black Sea, Mediterranean Sea and the Northeastern Atlantic waters from Morocco to the Cabo Blanco peninsula (Karamanlidis et al., in press), its populations have been drastically reduced and

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fragmented by human exploitation in prehistoric and historic times (Johnson and Lavigne, 1999; Johnson, 2004; Stringer et al., 2008; Brito, 2012; González, 2015) and, more recently, by adverse interactions with fishermen and coastal development (Karamanlidis et al., in press). The current range of the Mediterranean monk seal is extremely patchy and only a fraction of its original prehistoric range. It consists of three, maybe four, geographically isolated subpopulations (Fig. 1): In the

Mediterranean Sea, the vast majority of monk seals live in the Ionian and Aegean Seas, and along the coasts of mainland Greece, Cyprus and western and southern Turkey (Gücü et al., 2004, 2009; Güçlüsoy et al., 2004; Anonymous, 2007). An unknown number of Mediterranean monk seals, likely fewer than ten, might still survive at the Mediterranean coasts of eastern Morocco and Algeria (Mo et al., 2011), but without ongoing systematic monitoring and conservation actions the status and

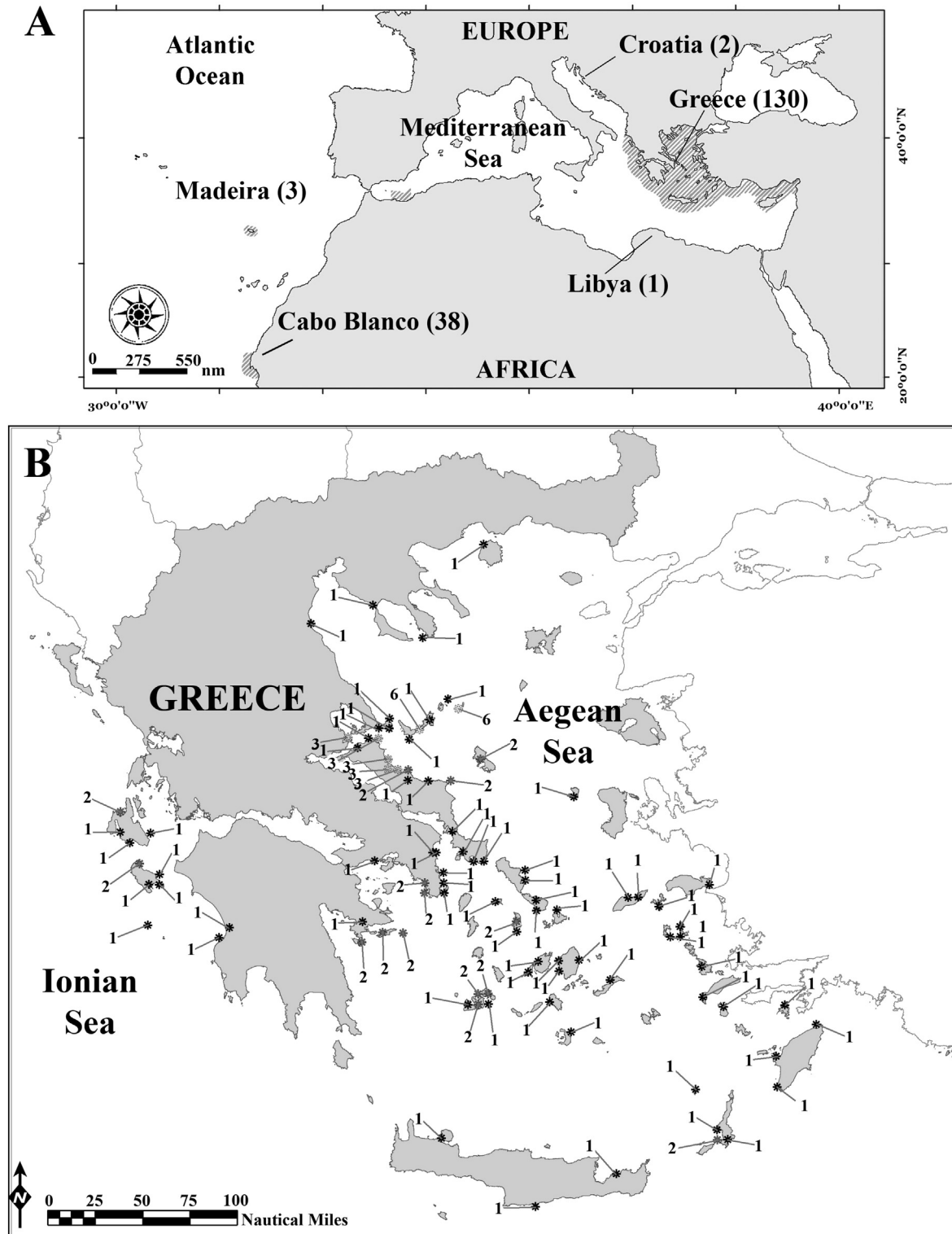


Fig. 1. Map of the Mediterranean and northern Atlantic Sea (A) and Greece (B) indicating the locations and number of genetic samples collected from Mediterranean monk seals (1990–2012). The shaded areas in map A indicate the extant distribution of the Mediterranean monk seal.

fate of this subpopulation are in question. In the North Atlantic, two subpopulations exist: one at the Cabo Blanco peninsula, straddling the northern border of Mauritania and the southern fringe of the Western Sahara (González et al., 1997; González and Fernandez de Larrinoa, 2012; Martínez-Jauregui et al., 2012), and another at the Archipelago of Madeira (Pires et al., 2008). In recent years several extra-limital sightings of vagrant animals have been recorded throughout the eastern Mediterranean Sea, reaching as far as Libya, Croatia and the Balearic Islands (Karamanlidis et al., in press). Overall, the Mediterranean monk seal has been decimated and total abundance is currently estimated to number fewer than 700 individuals: approximately 350–450 individuals live in the eastern Mediterranean Sea (i.e., Greece, Cyprus and Turkey), 250 at the Cabo Blanco peninsula and fewer than 50 in the Archipelago of Madeira (Karamanlidis et al., in press). The monk seal subpopulation at Cabo Blanco in the Atlantic (Martínez-Jauregui et al., 2012) and the monk seals at Gyaros Island in the eastern Mediterranean (Karamanlidis et al., 2013) are the only large extant aggregations of the species that still preserve the structure of a colony; all other subpopulations in the eastern Mediterranean and at Madeira are usually small, fragmented groups of <20 individuals.

Partly due to the intensity of human persecution Mediterranean monk seals now use remote, inaccessible marine caves for resting and pupping (González, 2015; Karamanlidis et al., in press), making studying and understanding the biology of an already rare species even more difficult. Considering our poor understanding of the species biology and its critically endangered status, information on the genetics of the Mediterranean monk seal should be considered imperative for the design and implementation of effective management and conservation actions (Kovacs et al., 2012). The population reduction(s) that the species has undergone, the severity and duration of which are unknown, have likely reduced its genetic diversity through genetic drift and founder effects (Pastor et al., 2007). Studying the genetic diversity of poorly understood, endangered species is important for their conservation planning, as their diversity measures can be compared with diversity levels of populations with detailed information on population history and status (Kretzmann et al., 1997; Johnson et al., 2009).

The aim of this study was to describe the genetic diversity and population structure of the Mediterranean monk seal. We studied patterns of variation in the sequences of the control region of the mitochondrial DNA (mtDNA) to gain insights into the population history and dynamics of this poorly understood species. Through mtDNA analysis of living organisms, we can make a significant contribution to the long-term planning and short-term execution of endangered species recovery plans (Moritz, 1994), which was our study's ultimate goal.

2. Material and methods

2.1. Sample collection

We collected samples (i.e., tissue, scat, hair, bone, dried milk) from Mediterranean monk seals during population monitoring efforts carried out from 1990 to 2012. In total, 174 samples from individual seals were collected. Samples originated from all three major monk seal subpopulations [i.e., Aegean ($N = 117$) and Ionian Seas in Greece ($N = 13$), Madeira ($N = 3$), Cabo Blanco peninsula ($N = 38$)] and from vagrant animals in Croatia ($N = 2$) and Libya ($N = 1$), thus covering the entire extant range of the species (Fig. 1).

2.2. DNA extraction and molecular analysis

Tissue samples were stored in 95% ethanol or DMSO at -20°C . DNA from tissue, hair, bone, and milk was extracted using the Qiagen DNeasy® Blood & Tissue Kit following the manufacturer's protocol. DNA from scat was extracted using the Qiagen QIAamp® DNA Stool Mini Kit following a protocol optimized for carnivore scat extraction (Caragiulo et al., 2014).

Previous studies by Pastor et al. (2004, 2007) had found very low diversity at nuclear microsatellite loci. Suspecting that mitochondrial genetic diversity would likewise be low, we chose to examine the mitochondrial control region, which has the highest intraspecific diversity levels of any region in the mitochondrial genome. For twelve tissue samples from the Ionian Sea, the northern Aegean Sea and the southern Aegean Sea, the entire mitochondrial control region was amplified using the primers MMOCR-F (TTC CCC GGT GTA AAC C) and MMOCR-R (ATT TTC AGT GTC TTG CTT T) (Goldsworthy et al., 2000) and 12 full sequences of ~1300 base pairs (bp) were obtained and aligned with each other. A region of the d-loop was identified that showed variation between three of the sequences. Due to a low success rate in amplifying the ~1300 bp fragment, primers were designed to isolate a shorter fragment of the hypervariable region I (HVR1) that included the observed variations. Using Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012) in the software program Geneious v. 6.1.7 (Biomatters, www.geneious.com, Kearse et al., 2012) forward (MMS_dlp564-F: CCC GCG GCC TAT GTA ACT CG) and reverse (MMS_dlp564-R: TGC GCC TCA TGG TTG TAT GA) primers were designed to isolate the shorter variable region.

In a pre-PCR product lab, a reaction was set up using a recipe of 21.5 μL deionized water, 1.0 μL forward primer (10 mM), 1.0 μL reverse primer (10 mM), 1 PuReTaq Ready-to-Go™ PCR bead (GE Healthcare), and 1.5 μL DNA template. DNA was amplified with 35 cycles using the thermocycler protocol: 94.0°C for 30s, 56.5°C for 30s, and extension at 72.0°C for 1 m, followed by a final extension at 72°C for 10 min. All reactions were run on a 1.5% Agarose gel to check for proper sized bands. PCR products were then purified using AMPure® XP (Agencourt®) and cycle-sequenced in both directions using a recipe of 4.75 μL deionized water, 0.5 μL BigDye® (Applied Biosystems®), 0.75 μL Extension Buffer (Applied Biosystems®), 1.0 μL primer (3 mM), and 1.0 μL purified PCR product. Cycle-sequencing products were cleaned using CleanSeq® (Agencourt®) and eluted in 25 μL of EDTA. Sequences were run on an ABI 3730xl DNA analyzer (Applied Biosystems®).

2.3. Data analysis

Forward and reverse sequences were aligned and edited using the program Sequencher v. 5.0.1 (Gene Codes Corporation). Sequences of the proper length (524 bp) and of high quality (>85% of nucleotides were called with high confidence) were accepted and aligned using the Geneious pairwise alignment algorithm in Geneious Pro v. 6.1.7 (Biomatters, www.geneious.com, Kearse et al., 2012) to compare for variation. Failed samples were repeated at least once from the PCR stage. All unique haplotypes were verified by independent reactions to ensure that polymorphisms were not PCR artifacts.

We evaluated genetic diversity by calculating haplotype frequencies and distances between haplotypes using the program Arlequin 3.1 (Excoffier et al., 2005). We used the same program to estimate haplotype and nucleotide diversities (Nei, 1987).

Due to the small sample size in Madeira, Croatia and Libya and based on geographic distances, we evaluated the genetic structure of the remaining Mediterranean monk seal subpopulations, by a priori separating and comparing only the samples from the Ionian Sea, Aegean Sea, and Cabo Blanco. We used the program Arlequin to calculate haplotype diversity (h) and nucleotide diversity (π) of the species and each regional group, as well as F_{ST} values and exact tests between the regions. Using Fisher's exact tests we checked for genetic differentiation also within the Aegean Sea, by a priori separating it, based again on geographic distances, into three separate regions: Northern Aegean Sea ($N = 47$, i.e., Northern Sporades islands and island of Evoia), Central Aegean ($N = 42$, i.e., Attica and Cyclades Islands) and Southern Aegean Sea ($N = 21$, i.e., Dodecanese islands).

In order to evaluate the phylogeography of the studied populations, Mediterranean monk seal sequences were aligned with a control

region sequence of the mitochondrial genome from the closest relative of the species, the Hawaiian monk seal (*Neomonachus schauinslandi*) (GenBank accession: AM181022). A haplotype network of the Mediterranean monk seal sequences was created using the program TCS v. 1.21 (Clement et al., 2000).

3. Results

The full mtDNA control region, including the short tandem repeat (STR) region of the mammalian mitochondrial d-loop was amplified in 12 samples. Using primers designed from these sequences, we successfully amplified a 524 bp fragment of the hypervariable mtDNA control region for 165 of the 174 samples: 121 samples were amplified from Greece, 2 from Croatia, 1 from Libya, 3 from Madeira, and 38 from the Cabo Blanco peninsula. Monk seal control region sequence variation was very low; of the 524 sites surveyed, only 3 were polymorphic. The 3 variable sites defined 5 haplotypes (named MM01–MM05, GenBank Accession numbers KT935307–KT935311). All polymorphisms were a result of a transition substitution (adenine to guanine or vice versa).

The haplotype MM01 was the most common and was found in 82 individuals (68% of samples from Greece) in both the Aegean and Ionian Seas, as well as in the vagrants from Croatia ($N = 2$) and Libya ($N = 1$). All other haplotypes, however, were endemic to geographically distinct regions: Haplotype MM02 was found in 28 individuals (23% of samples from Greece), exclusively in the Aegean Sea; Haplotype MM03 was found in 10 individuals (8.2% of samples from Greece), exclusively in the Ionian Sea (83.3% of samples from the Ionian Islands); the rarest haplotype, MM04, was only found in 1 individual from the island of Karpathos in the Aegean Sea (Table 1), one of the easternmost sampling locations in Greece. The PCR and sequencing for this sample was repeated twice to ensure that this polymorphism was not a PCR artifact. All seals from Madeira ($N = 3$) and the Cabo Blanco peninsula ($N = 38$) carried a single haplotype, MM05, not found in any seal in the eastern Mediterranean Sea.

3.1. Genetic diversity

The five haplotypes ranged 99.4–99.8% in similarity. Haplotype diversity (h) of the subpopulation in Greece was $0.481 (\pm 0.042)$, while its nucleotide diversity (π) was $0.001 (\pm 0.001)$. The Aegean Sea sampling area had the highest diversity, with a haplotype diversity of $0.378 (\pm 0.042)$ and a nucleotide diversity of $0.001 (\pm 0.001)$. The Ionian Sea sampling area had lower diversity, with $h = 0.303 (\pm 0.147)$ and $\pi = 0.001 (\pm 0.001)$. All other sampling areas showed only one haplotype, resulting in zero haplotype diversity and nucleotide diversity.

3.2. Population structure

The pairwise F_{ST} analysis revealed significant differences between the Aegean and Ionian sampling areas ($F_{ST} = 0.577$; $P = 0.000$), as did

an exact test of population differentiation ($P = 0.000$). The Cabo Blanco sampling area was found to be distinct from both the Aegean ($F_{ST} = 0.733$; $P = 0.000$) and the Ionian Sea ($F_{ST} = 0.925$; $P = 0.000$) sampling areas. There was evidence of population differentiation also within the Aegean Sea between the three a priori groupings of islands (Fisher's Exact Test, $P = 0.019$). While there was no difference between the North and the Central Aegean (Fisher's Exact Test, $P = 0.623$), we detected a difference between the Southern Aegean and both the Central (Fisher's Exact Test, $P = 0.044$) and Northern Aegean Sea (Fisher's Exact Test, $P = 0.007$). In fact, MM02 was not detected in the Southern Aegean (Table 2, Fig. 2), which would be unlikely if its frequency in the area was the same as in the rest of the Aegean ($P = 0.010$).

3.3. Population history

A network of the five haplotypes identified showed that each haplotype differed by only one nucleotide from another haplotype (Fig. 3). Three of the five haplotypes (MM02, MM03, MM05) differed from a single haplotype (MM01) by one nucleotide. This combined with the fact that MM01 was the most common and widespread haplotype in the Mediterranean, suggests that MM01 is the ancestral haplotype of the Mediterranean monk seal. Furthermore, the nucleotides at all three polymorphic sites among Mediterranean monk seal haplotypes were the same in MM01 and the available Hawaiian monk seal reference sequence, while all other Mediterranean monk seal haplotypes differed from the Hawaiian monk seal sequence at one or two of those sites. This indicates that the MM01 sequence is ancestral and the other four Mediterranean seal haplotypes have arisen through mutations at the polymorphic sites. All haplotypes differed by only one nucleotide from at least one other haplotype in the network, indicating furthermore that there are no intermediate haplotypes missing from the network.

4. Discussion

This study reveals that mitochondrial DNA sequence analysis can be a useful tool in acquiring baseline information on the biology of elusive, critically endangered species. The present study confirmed previous indications that the extant population of the critically endangered Mediterranean monk seal has low genetic diversity (Pastor et al., 2004, 2007), as only five haplotypes were found throughout the entire extant range of the species. It also clearly identified for the first time three genetically distinct subpopulations: one in the North Atlantic Ocean and two in the Mediterranean (one in the Ionian and another one in the Aegean Sea).

4.1. Genetic diversity

Due to its role as the raw material for natural selection (Schultz, 2011) and the expected relationship between heterozygosity and population fitness (Frankham, 1995), genetic diversity has been identified by the IUCN as an important form of biodiversity, deserving conservation within each population (Reed and Frankham, 2003). Although we discovered at least three genetically distinct subpopulations, haplotypic diversity within each Mediterranean monk seal subpopulation and within the species overall was low. Monk seal haplotypic diversity was similar to that found in other bottlenecked seals [e.g. Hawaiian

Table 1
Distribution of Mediterranean monk seal mtDNA control region haplotypes by sampling area.

Haplotype	Sampling area						
	Mediterranean Sea				North Atlantic		
	Croatia	Greece		Libya	Cabo Blanco	Madeira	Total (%)
		Aegean Sea	Ionian Sea				
MM01	2	80	2	1	0	0	85 (51)
MM02	0	28	0	0	0	0	28 (17)
MM03	0	0	10	0	0	0	10 (6)
MM04	0	1	0	0	0	0	1 (0.6)
MM05	0	0	0	0	38	3	41 (25)
Total	2	109	12	1	38	3	165

Table 2
Number of monk seal samples with MM01 and MM02 haplotypes in different areas of the Aegean Sea.

Area	MM01 (N)	MM02 (N)	Total
Northern Aegean	31	14	45
Central Aegean	28	9	37
Southern Aegean	17	0	17
Total	76	23	99

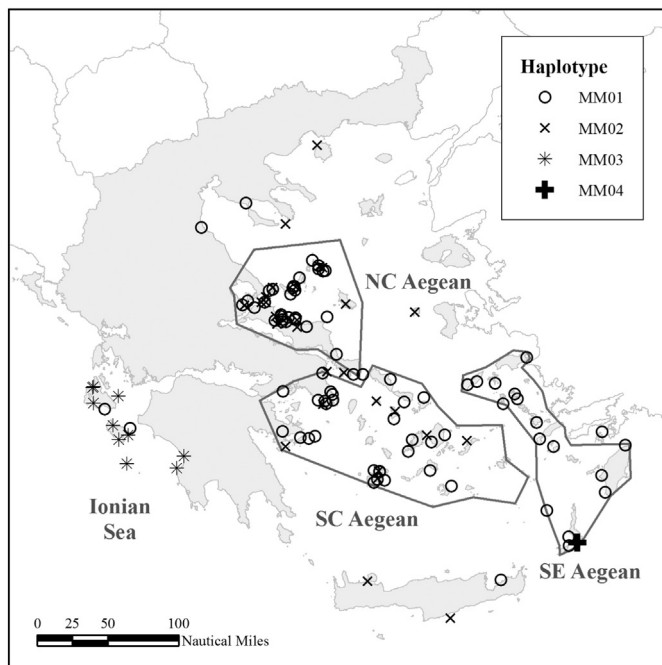


Fig. 2. Map of Mediterranean monk seal haplotypes in Greece by a-priori set areas. A small measure of random noise was added to locations to enhance visualization of samples collected at the same location.

monk seal (Kretzmann et al., 1997), Northern elephant seal (*Mirounga angustirostris*) (Hoelzel et al., 1993)], as well as in some terrestrial carnivores also threatened with extinction, such as the Ethiopian wolf (*Canis simensis*) (Gottelli et al., 2004) and the cheetah (*Acinonyx jubatus*) (Freeman et al., 2001; Charrau et al., 2011). Based on the number of haplotypes per individuals assessed and the number of variable sites in relation to the total number of sites sequenced, the Mediterranean

monk seal appears to be one of the most genetically depauperate mammals on Earth (Table 3). The low mtDNA diversity was in accordance with the low genetic diversity detected when using nuclear markers. Using a set of 24 microsatellite loci Pastor et al. (2004, 2007) found that as a consequence of severe bottlenecks, the monk seal populations in Cabo Blanco and in the eastern Mediterranean had suffered a decrease in genetic variability over the last few centuries. Also, F_{ST} values and the estimated number of migrants per generation indicated substantial genetic differentiation between monk seals in Cabo Blanco and Greece.

It is difficult to draw general conclusions and make predictions on the conservation prospects of the Mediterranean monk seal based solely on this finding (Lande, 1988; Caro and Laurenson, 1994; Merola, 1994; O'Brien, 1994), because mtDNA is maternally inherited and has a smaller effective population size than nuclear markers. Small populations may therefore lose mitochondrial variability while retaining nuclear diversity (Avise et al., 1985; Birky et al., 1989). Also, the mitochondrial control region is non-coding and therefore considered to not experience strong selective pressure. Diversity at such neutral loci is expected to be lost in decreasing populations more quickly than protein-coding loci under selective pressure (Amos and Balmford, 2001).

While lower genetic diversity in a population is suspected to endanger its viability, in pinnipeds this relationship is not clear. Northern elephant seals were thought to have been hunted to extinction in the late 19th century. From genetic analysis, it is estimated that as few as 20 animals survived the period of commercial harvesting (Campagna, 2008), but current estimates put the population at more than 200,000 individuals and growing (Lowry et al., 2014). A survey of mitochondrial genetic diversity in these seals found only two haplotypes in 40 seals with only 3 variable sites in 300 bp of the mitochondrial control region (Hoelzel et al., 1993). In contrast, other species that are known to have experienced bottlenecks during the heavy fad of seal hunting in the 18th and 19th centuries, such as the Antarctic fur seals (*Arctocephalus gazella*) and the Guadalupe fur seals (*Arctocephalus townsendi*), have made recoveries similar to that of the Northern elephant seal. These species of fur seals, however, retain significant mitochondrial diversity (Bernardi et al., 1998; Wynen et al., 2000). Finally, a study by Kretzmann et al. (1997) of control region variability in Hawaiian monk seals also found a low number of haplotypes (3 haplotypes in 50 animals); however Hawaiian monk seals have not shown significant signs of recovery and may currently be decreasing (Baker et al., 2011). It is therefore likely that factors involved in the biology and ecology of the Mediterranean monk seal, such as the existence of suitable resting and pupping habitat, are more relevant to population recovery—or lack thereof—than the level of genetic diversity.

4.2. Population structure

Knowledge on spatial and population structure is necessary for the effective assessment and management of animal populations (González-Suárez et al., 2009). In marine mammals this is not always easy to obtain because these animals occupy seemingly continuous and extensive habitats and are capable of dispersing over long distances (Campagna et al., 2001). For seals, in particular, some degree of spatial and population structure might be expected as they aggregate in colonies (Riedman, 1990) and often show high levels of philopatry (Pomeroy et al., 2000). In the present study three genetically distinct subpopulations of the Mediterranean monk seal were identified for the first time: one in the Atlantic and two in the eastern Mediterranean Sea, with the one in the Aegean indicating further, finer structure. Considering the site fidelity of Mediterranean monk seals (Gazo et al., 1999) and the straight distance of >1200 km between the closest Atlantic and Mediterranean seal subpopulations, the separation of these two areas and the existence of two genetically isolated subpopulations was to be expected and is in accordance with information from nuclear DNA studies (Pastor et al., 2007). However, the

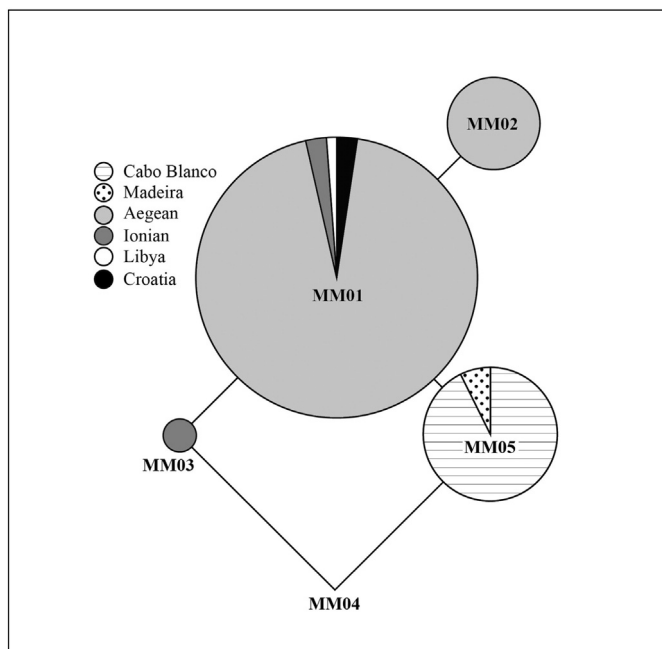


Fig. 3. Haplotype network of the five Mediterranean monk seal d-loop haplotypes by location, shown to scale. The MM04 haplotype was found in only one individual from the Aegean Sea.

Table 3
Intraspecific mtDNA control region sequence variation for pinnipeds and other endangered species.

Species	Haplotypes/individuals	Haplotypic diversity	No. variable sites/total no. sequenced	Variable sites %	Source
South American fur seals (<i>Arctocephalus australis</i>)	29/65	0.87	29/267	10.8	Crespo et al. (2015)
Galapagos fur seal (<i>Arctocephalus galapagoensis</i>)	14/87	0.86	14/220	6.4	Lopes et al. (2015)
Southern elephant seal (<i>Mirounga leonina</i>)	26/48	0.54	26/300	8.7%	Hoelzel et al. (1993)
Juan Fernández fur seal (<i>Arctocephalus philippii</i>)	13/28	0.46	39/313	12.5%	Goldsworthy et al. (2000)
Cheetah (<i>Acinonyx jubatus</i>)	8/20	0.4	27/525	5.1%	Freeman et al. (2001) and Charruau et al. (2011)
California sea lion (<i>Zalophus californianus</i>)	11/40	0.28	29/315	9.2%	Maldonado et al. (1995)
Guadalupe fur seal (<i>Arctocephalus townsendi</i>)	7/25	0.28	18/313	5.7%	Bernardi et al. (1998)
Steller sea lion (<i>Eumetopias jubatus</i>)	52/224	0.23	29/238	12.2%	Bickham et al. (1996)
Harbor seal (<i>Phoca vitulina</i>)	34/227	0.15	40/453	8.8%	Stanley et al. (1996)
Ethiopian wolf (<i>Canis simensis</i>)	10/66	0.15	10/231	4.3%	Gottelli et al. (2004)
Hawaiian monk seal (<i>Neomonachus schauinslandi</i>)	3/50	0.06	2/359	0.6%	Kretzmann et al. (1997)
Northern elephant seal (<i>Mirounga angustirostris</i>)	2/40	0.05	3/300	1.0%	Hoelzel et al. (1993)
Mediterranean monk seal (<i>Monachus monachus</i>)	5/165	0.03	3/524	0.6%	Present study

population differentiation observed between monk seals in the Ionian and the Aegean sea and within the Aegean Sea itself is more difficult to explain, considering that monk seals in these areas are known to travel distances that exceed the distance between these sampling areas (Adamantopoulou et al., 2011), thus making gene flow between them likely. Considering that mtDNA is maternally inherited (Avise et al., 1985) the population differentiation between the Ionian and Aegean Sea and within the Aegean Sea might be explained by the pronounced female philopatry of the species (Gazo et al., 1999). This in turn highlights the importance of the presence and the necessity for effective protection of suitable pupping habitat in the eastern Mediterranean. Along the same line is the presence of a single individual with a unique haplotype (i.e., MM04) at one of the easternmost sampling locations within the Aegean, which could possibly indicate the existence of another subpopulation in the eastern Mediterranean Sea, or traces of a formerly more diverse and structured population in this region. This genetic picture suggests a severe decrease in population size and fragmentation, where there was very limited gene flow between small remaining subpopulations and very fast genetic drift in each of them. The issue should be further explored using nuclear genetic markers and analysis of samples from the Mediterranean coasts of Turkey.

The Mediterranean monk seal subpopulation in the eastern Mediterranean Sea has recently been showing encouraging signs of population recovery. Evidence for this recovery is provided by systematic monitoring efforts in the main pupping sites in Greece, the information of the Hellenic National Monk Seal Rescue and Information Network (Anonymous, 2007), as well as numerous extra-limital sightings in the eastern Mediterranean Basin (Karamanlidis et al., in press). We believe that this recovery is responsible for the presence of vagrant individuals, with the most common mtDNA haplotype found in Greece (i.e., haplotype MM01, which is found in both the Ionian and Aegean sampling areas), in Libya and Croatia, areas where the species is believed to have been effectively extinct (Gomerčić et al., 2011; Alfaghi et al., 2013).

The persistence of small, isolated subpopulations and low inter-population migration rates have likely helped the Mediterranean monk seal to preserve more genetic variation than would be expected in a fully panmictic population (Varvio et al., 1986; Lacy, 1987). In comparison, the Hawaiian monk seal population, which is largely panmictic and whose size is almost one and a half times that of the Mediterranean monk seal (Schultz et al., 2011), has similarly low levels of genetic diversity (Kretzmann et al., 1997).

4.3. Population history

The haplotype network generated here (Fig. 3), the analysis of haplotype distribution, as well as the comparison of nucleotide polymorphisms to a *N. schauinslandi* reference sequence, all support MM01 as the ancestral haplotype in Mediterranean monk seals. It is impossible to determine the exact location of evolution of the species based on this information, but given the extremely close relationship of the five haplotypes detected, the expansion of the distribution of the species appears to be a relatively recent evolutionary event, as slower and more ancient colonization events usually would allow for more genetic diversification (Avise, 2000).

The lack of missing haplotypes and the single nucleotide differences between the five haplotypes cannot be explained simply by an anthropogenic bottleneck of a species that has existed in place over significant evolutionary time. Recent studies have estimated that the split between Mediterranean monk seals and the Neotropical lineage occurred around 6 million years ago (Fulton and Strobeck, 2010; Scheel et al., 2014). If the Mediterranean monk seal population had been consistently large up until the recent human-caused decline, many more mutations would have been expected to accumulate in the mitochondrial control region. The human-induced population bottleneck could have brought the number of haplotypes in the species down to five, but because haplotypes would have been lost at random this would result in intermediate haplotypes missing from the network.

Rather, alternative hypotheses are required to explain the observed pattern of the low levels of mitochondrial diversity and closely related haplotypes. One possibility is that in recent evolutionary history, Mediterranean monk seals experienced at least one bottleneck from which they subsequently expanded through their current range. During this time, if a metapopulation structure existed this may also have maintained lower levels of differentiation populations throughout the range. Following this expansion, the population has declined over the past 2,000 years, due to human persecution and habitat alteration (Karamanlidis et al., in press), which led to the current low levels of genetic diversity through smaller population size and genetic drift.

4.4. Implications for research and conservation

The Mediterranean monk seal is currently one of the most endangered marine mammals on Earth and urgent and effective conservation

actions are necessary to safeguard its future. Due to inherent difficulties in studying Mediterranean monk seals, the biology of the species continues to be poorly understood, a fact that is considered to have hampered its effective conservation (Johnson et al., 2006) and allowed for conservation planning only on a local [i.e., Species Action Plans in Greece and Turkey (Notarbartolo di Sciara et al., 2009; Kiraç et al., 2013)] or regional scale [i.e. Species Action Plans in the eastern Atlantic and the eastern Mediterranean (González et al., 2006; Notarbartolo di Sciara, 2013)]. This study is the first range-wide study of the species and provides baseline information that will help identify research and management priorities for designing a comprehensive conservation strategy for the species as a whole.

On the research level, this study has been made possible due to the systematic, long-term monitoring efforts carried out throughout the species' range and the collection and storage of samples in dedicated sample banks. Given the fact that these sample banks have provided the material for this study and for studies analyzing microsatellite alleles (Karamanlidis et al., 2014) and major histocompatibility complex genes (Gaughran, 2013) that will further our understanding of the population substructure, level of inbreeding, and species-level diversity at important immune genes, and acknowledging the fact that all monk seal subpopulations are important for the survival of the species, it is imperative that the systematic monitoring efforts for the species continue and efforts to collect genetic samples intensify. Genetic samples should be collected during necropsies from all dead animals and from live animals found stranded and/or treated in rehabilitation. Non-invasive genetic samples (i.e., scat and shed hair) should be collected systematically during regular cave visits.

Although a lack of genetic diversity may not always be of foremost concern in conservation, its potential relevance to the prospects of an endangered species should not be underestimated. Low genetic variability may increase vulnerability of a species to infectious disease (Bonnell and Selander, 1974; O'Brien and Evermann, 1988) and may have devastating effects in a small population. The effects of stochastic events on the demographic trajectory of a species were demonstrated vividly in the Mediterranean monk seal in 1997, when a mass die-off of seals at the Cabo Blanco peninsula wiped out two thirds of the entire colony (Forcada et al., 1999). Also, inbreeding depression, which like low genetic diversity is often a product of small population sizes or bottlenecks (Hedrick and Kalinowski, 2000), might threaten the survival of local monk seal populations. The monk seal's low natality rates (Gazo et al., 1998; Gücü et al., 2004), a common result of inbreeding depression, represents a real threat to the monk seal's recovery, and a genetic basis for this poor reproductive performance cannot be ruled out. As previously undetected inbreeding depression may be manifested in the face of environmental challenges (Jiménez et al., 1994; Keller et al., 1994), all monk seal populations should be closely monitored genetically.

On a management level the existence of two genetically distinct monk seal subpopulations in the Atlantic and in the Mediterranean Sea (with no sharing of haplotypes and limited gene flow), in combination with marked differences between the two subpopulations in demographics and ecology and behavior (Karamanlidis et al., in press), justify the until recently de facto conservation of these units as separate management units; this separation was based on geographical distance and political and legislative criteria. Furthermore, the existence of at least two distinct monk seal subpopulations in Greece (i.e., one in the Ionian and one in the Aegean Sea) will provide new challenges to the conservation of the species in the region. It has been suggested that a functional network of marine protected areas will be necessary to safeguard the future of the species in the country (Adamantopoulou et al., 2000). The information on population structure and the inferences made regarding the site fidelity of reproductive females which highlight the importance of suitable pupping habitat should be complemented by information on habitat availability and suitability throughout the country and integrated in the protected area planning for the species that is underway.

Currently, detailed information on habitat availability and suitability for the monk seal throughout entire Greece does not exist; this has been identified as an important conservation priority for the species in the country (Notarbartolo di Sciara et al., 2009), but has not been implemented yet. Furthermore, cooperation between Greece and Turkey should be intensified and genetic samples from the Mediterranean coasts of Turkey should be analyzed in order to elucidate the potential finer population sub-structuring of the Mediterranean monk seal in the eastern Mediterranean.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.biocon.2015.11.014>.

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