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INCIDENTAL CATCH OF LOGGERHEAD SEA TURTLES (*CARETTA CARETTA*) IN THE MEDITERRANEAN: CONSERVATION CONSEQUENCES OF GENETIC HETEROGENEITY BETWEEN FORAGING GROUNDS

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FOREWORD

The present work aims at defining the foraging grounds exploited by immature Mediterranean Loggerhead turtles from different subpopulations and specially to distinguish them from the ones mostly used by Atlantic individuals, should this spatial segregation exist.

Although the project is still ongoing and the final version will be ready at the end of 2005, there are already important results relevant to turtle conservation. Therefore the Secretariat has considered important to present to the Parties these preliminary results, in order to allow them considering the conclusions so far obtained within their future management actions for the loggerhead turtle in the Mediterranean.

A very methodical thorough work in the field of biological conservation research has been successfully implemented, in order to achieve the genetic identification of turtles by-caught in the Mediterranean and that of the Mediterranean breeding colonies. Spatial analysis of the findings has allowed obtaining sound conclusions related to the priorities where conservation efforts should be concentrated, regarding interaction with fisheries and vulnerability of different turtle breeding beaches. The spatial matching of these findings with fishing activities by-catching turtles will point out defined areas and fishing fleets to be considered in future priority management actions les;

Further analysis carried out along 2005 will aid to obtain a more precise image of the situation regarding additional samples.

Abstract

This report analyses the genetic structure of nesting sites in the eastern Mediterranean and that of foraging grounds in the central and western Mediterranean to assess the relevance of the incidental catch of loggerhead sea turtles on the Mediterranean population.

The genetic structure of the Mediterranean nesting populations was assessed by means of a mitochondrial DNA marker and seven microsatellites. Genetic structuring was revealed by both kinds of markers, although male-mediated gene flow exists between some populations for nuclear DNA. Private haplotypes were found in Greece and Israel. Furthermore, some females nesting in Turkish beaches carry a haplotype not found anywhere else in the Mediterranean, although reported from Atlantic nesting beaches. Crete and Cyprus are thought to play a central role in the male-mediated gene flow between Mediterranean populations and due to these connections, the negative effect of genetic drift or inbreeding on the smallest populations of Lebanon and Israel may be less important than those suggested by its reduced population size.

The analysis of mitochondrial DNA from eight feeding grounds in the Mediterranean and the adjoining Atlantic revealed deep genetic structuring. As a consequence, the foraging grounds off the northern African coast and the Gimnesies Islands are inhabited mainly by turtles of the Atlantic stocks, whereas the foraging grounds off the European shore of the western basin, and those in the eastern basin, are thought to be inhabited mainly by turtles from the eastern Mediterranean rookeries. Finally, the foraging grounds off Lampedusa and Pitiüses islands are to be inhabited by specimens of both origins. This structuring is explained by the pattern of sea surface currents and water masses and suggests that immature loggerhead sea turtles entering the western Mediterranean from the Atlantic and the eastern Mediterranean remain linked to particular water masses, with a limited exchange of turtles between water masses. As the foraging grounds off the European shore of the western Mediterranean comprise almost entirely individuals from the highly endangered eastern Mediterranean rookeries, conservation plans should make it a priority to reduce the mortality caused by incidental by-catch in these areas. Conversely,

private alleles from Mexico have been found in foraging grounds off the Balearic archipelago, thus suggesting that incidental by-catch in the Algerian basin may negatively affect the small populations in the Caribbean.

Introduction

The loggerhead sea turtle (*Caretta caretta*) is the most common sea turtle in the Mediterranean Sea (Broderick *et al.* 2002). Although sporadic nesting has been reported from the western basin (Tomas *et al.* 2002, Llorente *et al.* 1992, Delauguerre and Cesarini 2004), most of the nesting beaches are located in the eastern basin (Margaritoulis *et al.* 2003). Previous genetic studies (Bowen *et al.* 1993, Encalada *et al.* 1998) have shown that the population nesting in the Mediterranean becomes isolated from the Atlantic parental populations at the beginning of the Holocene, hence suggesting that the Mediterranean population should be considered as a management unit independent of the Atlantic ones. The Mediterranean population has declined due to incidental catch, egg harvest and tourism development (Margaritoulis *et al.* 2003) and thus Groombridge (1990) recommended that the species should be considered critically endangered there. Genetic isolation from the Atlantic populations increases even more the vulnerability of the Mediterranean population, not only due to stochastic phenomena but also due to inbreeding depression and loss of genetic diversity due to genetic drift. Furthermore, the possible existence of an internal genetic structure within the Mediterranean may make things even worse, as several different management units might exist, some of them extremely small (Margaritoulis *et al.* 2003) and hence highly vulnerable.

Immature loggerhead sea turtles from Atlantic and eastern Mediterranean rookeries apparently share foraging areas in the western Mediterranean (Laurent *et al.* 1993, 1998; Laurent and Lescure, 1995; Casale *et al.* 2002). The individuals born in the Atlantic are thought to travel along the Gulf Stream, pass close to the Azores and Madeira and enter the western Mediterranean through the Strait of Gibraltar (Bolten 2003). The route followed by those coming from the eastern Mediterranean rookeries remains unknown, although adults use the Strait of Messina and the Sicilian channel to reach the western basin (Bentivegna 2002). Once in the western Mediterranean, little is known about how eastern Mediterranean and Atlantic juvenile turtles mix (Camiñas and de la Serna 1995) before returning to their beaches of origin for breeding (Plotkin 2003).

Several thousands of late juvenile loggerhead turtles are by-caught every year in the western Mediterranean by professional fishermen, mainly drifting long-liners (Mayolet *al.* 1988; Aguilaret *al.* 1995; Camiñas and de la Serna, 1995) but also drifting gill-nets (Silvaniet *al.* 1999) and trammel-nets (Carreras *et al.* 2004). Loggerhead sea turtles are also incidentally caught in the eastern Mediterranean (Godley *et al.* 1998; Casale *et al.* 2004), but total catch is unknown. Demographic studies indicate that the loss of late juveniles (straight carapace length from 30 to 80 cm) and adults has a more dramatic impact on populations than the loss of younger individuals (eggs, hatchlings and younger juveniles) (Crouse *et al.* 1987; NRC, 1990). Therefore, although rookery protection is a priority for marine turtle conservation, this measure will be unsuccessful without effective protection of large juveniles and adults. Boat collision, ingestion of debris, and chemical pollution are potential threats for larger size classes at sea (Lutcavage *et al.* 1997), but available data clearly indicates that fishing is the largest cause of mortality. Indeed, several declining populations have rapidly recovered once mortality caused by this activity was reduced (NMFS-SEFSC, 2001).

Previous genetic studies have considered a homogenous admixture of Atlantic and Mediterranean loggerhead sea turtles in the foraging grounds of the western Mediterranean (Laurent *et al.* 1993, Laurent *et al.* 1998; Casale *et al.* 2002) and hence incidental by-catch everywhere within the basin was thought to threaten the small and endangered populations nesting in the eastern Mediterranean. However, the western Mediterranean is topographically heterogeneous and is divided in a number of sub-basins characterized by different water masses and connected by two major surface currents. As loggerhead sea turtles of the Atlantic and the Mediterranean stocks gain access to the western Mediterranean using different routes, a passive drift is thought to play a relevant role in the dispersal of immature loggerhead sea turtles (Davenport and Clough 1986, Bolten 2003), an heterogeneous distribution of both type of loggerhead sea turtles within the western Mediterranean is expected, in agreement with the major current systems. Satellite tracking has revealed that loggerhead sea turtles in the Tyrrhenian Sea migrate seasonally to the eastern Mediterranean (Bentivegna 2002) but seldom enter the adjoining Algerian basin. Conversely, loggerhead sea turtles in the Algerian basin

sometimes enter the adjoining Balearic Sea, but not the Tyrrhenian Sea (Cardona *et al.* in press). Thus, exchange between sub-basins seems to be limited on the short run, which may lead to deep genetic structuring. In such a scenario, efforts to reduce by-catch should focus in the areas inhabited mainly by Mediterranean loggerhead sea turtles, as the impact of by-catch mortality on the much larger North-American population is probably negligible.

However, the eastern Mediterranean rookeries cannot be treated as a single unit, as available information suggests the existence of regional genetic structure not only for mitochondrial DNA markers, or mDNA (Laurent *et al.* 1993, Laurent *et al.* 1998, Encalada *et al.* 1998, Schroth *et al.* 1996), but also for nuclear DNA markers, or nDNA (Schroth *et al.* 1996). Unfortunately, sample size was often extremely low to reach sound conclusions and some important areas were not covered.

These report aims to (1) analyze the genetic structure of the nesting beaches in the eastern Mediterranean and (2) to assess the relative contribution of Atlantic and eastern Mediterranean populations to the foraging grounds in the central and western Mediterranean.

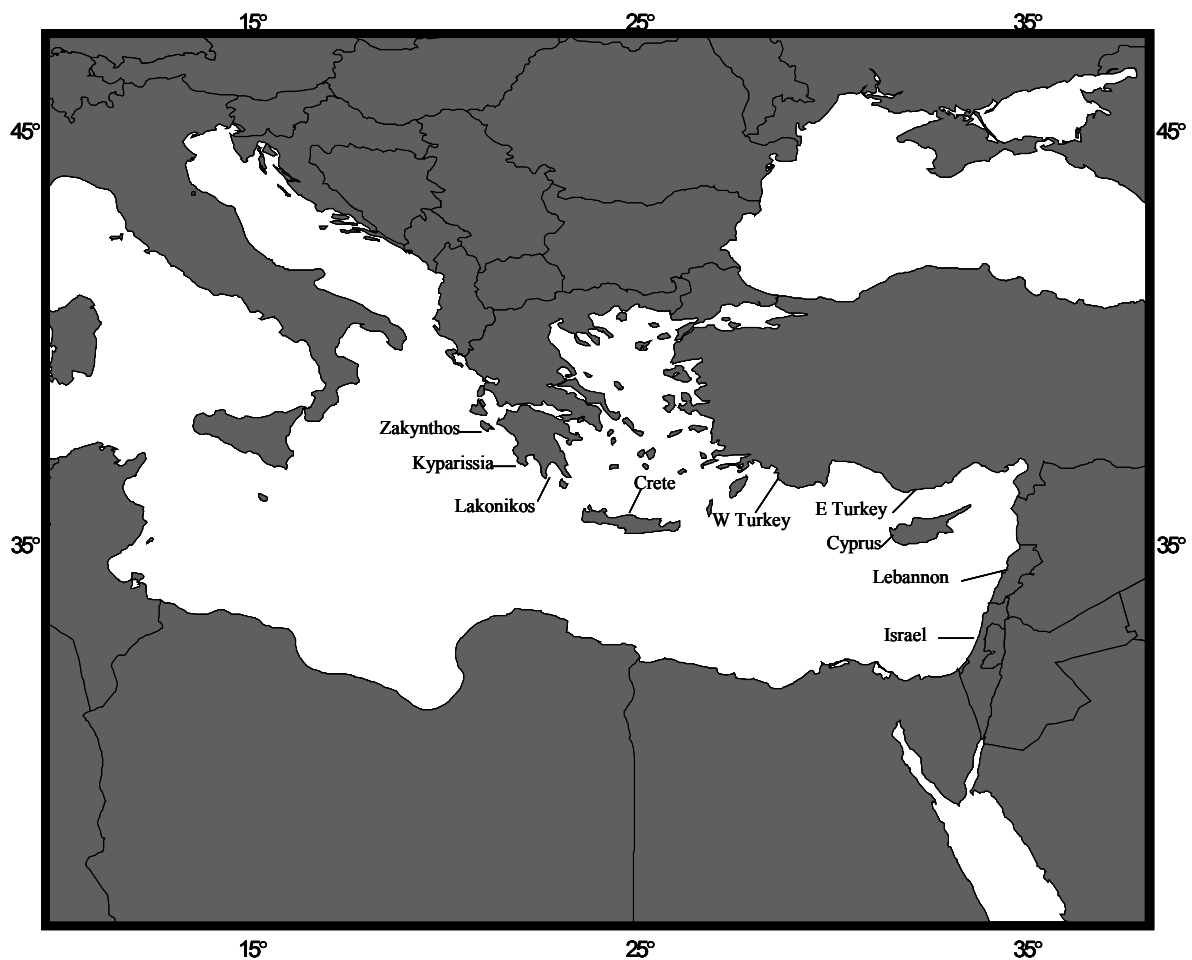
Material and Methods

Sample collection and DNA extraction

The analysis of the genetic structure of nesting sites in the eastern Mediterranean was based on tissue samples collected from 117 nests from 8 different nesting areas in the Mediterranean (Greece: Zakynthos, Lakonikos and Rethymnou, the later in Crete; Turkey: Fethiye, in the southwest; Lebanon: Tyre; north Cyprus; Israel) during the 2003 and 2004 nesting seasons. Previous data from the Greek population of Kyparissia (Encalada *et al.* 1998), not sampled in the present study, were also included for statistical analysis. Moreover, previous data from the populations of Cyprus and Turkey (Laurent *et al.* 1998) were also included, as the sampling size used in the previous studies were higher than the sample size of the present study. Sampling sites are shown in Figure 1.

When possible, each female was tagged while nesting (with metal flipper tags or subcutaneous PIT tags) to avoid pseudoreplication. When tagging was impossible, samples were collected only from clutches laid within a 15 day-window, as females usually nest more than one time in a season but rarely at intervals shorter than this period (Dutton 1995). Following this procedures, independency of the samples was ensured. Furthermore, only samples from one dead embryo/hatchling from each nest were analyzed. Muscle or skin samples were collected from dead embryos/hatchlings and stored in 95% ethanol. DNA was extracted using the QIAamp extraction kit (QIAGEN®) following the manufacturer's instructions.

Figure 1: Rookeries sampled in the eastern Mediterranean



The analysis of the genetic structure in the foraging grounds of the central and western Mediterranean was based on blood and tissue samples

from 282 loggerhead sea turtles, ranging in curved carapace length from 18 to 77 cm, collected from five locations: Gimnesies Islands (NE Balearic Islands), Pitiüses Islands (SW Balearic Islands), north-eastern Spain (from Ebro delta to the French border), western Italy (from the Strait of Messina to Cape Circeo) and eastern Italy (from the Strait of Messina to Campomarino).

Samples were collected from live and dead individuals during the summer from 1999 to 2003. Live animals were caught accidentally by fishermen or collected while basking by scuba divers (Ehrhart and Ogren 2000). Blood was taken from the dorsal cervical sinus of live turtles (Owens and Ruiz 1980) and stored at -20°C or preserved in lithium heparin at 4°C . Muscle or skin samples were collected from stranded animals and stored in a 20% DMSO NaCl 5M solution or stored at -20°C . All turtles were tagged with metal flipper tags or subcutaneous PIT tags before release to avoid pseudoreplication. Published data from foraging grounds off Lampedusa (Casale *et al.* 2002) and the Azores and Madeira (Bolten *et al.* 1998) were also included in the study since they are located between the western Mediterranean and the putative Atlantic and eastern Mediterranean nesting areas where the juveniles were born. Thus they are possibly intermediate points in turtle migration to the western Mediterranean.

Mitochondrial DNA analysis

A fragment of 391 base-pair (bp) of the control region of the D-loop of the mitochondrial DNA was amplified by polymerase chain reaction (PCR) using the primer pairs TCR1-TCR2, TCR5-TCR6 (Norman *et al.* 1994) or L71-H599 (Laurent *et al.* 1998). Sequences were aligned using the BioEdit package version 5.0.9 (Hall 1999) and compared with the previously found haplotypes (Bolten *et al.* 1998, Encalada *et al.* 1998, Carreras unpublished data), described in <http://accstr.ufl.edu/>. New haplotypes were assigned a code following the established nomenclature.

Genetic differentiation between different sampling sets of the same rookery was assessed with the Chi square test (Cuadras 1983). Values were compared to distributions obtained by randomizing individuals among populations using Monte-Carlo resampling (Rolf and Bentzen 1989) as

implemented in the program CHIRXC (Zaykin and Pudovkin 1993), and the Zs* test (Hudson *et al.* 1992) using the program DNAsp version 4.0 (Rozas *et al.* 2003). When statistically differences were not found between published and present data of the same nesting area, the two sets were considered to be subsamples of the same population.

Haplotype diversity (H) and nucleotide diversity (π) of each nesting area was calculated using the program DNAsp (Rozas *et al.* 2003) as well as the genetic distance (Gamma_{st} Nei 1982) between each pair of populations. Moreover, differentiation among all population pairs within the Mediterranean was also assessed by the Chi-square and Zs* test. Sequential Bonferroni correction was not applied neither for mtDNA nor for nDNA pairwise comparisons, as some authors may suggest (Moran 2003, Cabin and Mitchell 2000, Perneger 1998).

Effective population size (Ne) was calculated by rearranging the neutral equilibrium expectation $\pi=4Ne\mu$ and using the mutation rate of 1.29×10^{-5} found for the D-loop of mitochondrial DNA of the fish *Ameiurus nebulosus* (Chen and Herbert 1999). The number of migrants (Nm) between each population pair was calculated from genetic distances with the equation

$$Nm = \frac{1}{2} \left(\frac{1}{\text{Gamma}_{st}} - 1 \right).$$

Heterogeneity of haplotype frequencies between foraging grounds was assessed by the Chi-square test (Zaykin and Pudovkin 1993) and the Snn test (Hudson 2000). Values were compared to the distributions observed by randomizing individuals among populations using Monte-Carlo resampling (Rolf and Bentzen 1989), as implemented in the CHIRXC programme (Zaykin and Pudovkin 1993). With this method haplotypes occurring at low absolute frequencies had not to be grouped. Genetic distance (Gamma_{st}) between feeding grounds was calculated using the DNAsp programme version 4.0 (Rozas *et al.* 2003), and used to reconstruct a neighbour-joining tree. Chi square and Snn tests were used to assess the validity of the tree branches. A Mantel test, performed with GENEPOP (Raymond and Rousset 1995) was performed in order to detect if the genetic distance between foraging grounds was correlated to the geographic distance.

Microsatellite analysis

Seven microsatellite loci previously described were used in this study: cm84, cc117, cm72 and Ei8 (FitzSimmons *et al.* 1995); cc141 and cc7 (FitzSimmons *et al.* 1996); and ccar176 (Moore and Ball 2002). However the reverse primer of the locus ccar176 was redesigned using the sequence of the original clone found in genbank (accession number AF333763) in order to yield a longer fragment (Forward: 5'-GGCTGGGTGTCCATAAAAGA-3' and new Reverse: 5'- CCCTAAGTAAAGATTGGCTGCT-3'). One primer of each pair was fluorescently labeled with NED, PET, VIC or 6-FAM.

Tests for departure from Hardy-Weinberg equilibrium and linkage disequilibrium between loci were conducted for the rookery data. P-values were calculated with a Markov chain randomization (Guo and Thompson 1992). Fisher's method, which assumes statistical independence across loci, was used to combine test results for allelic counts among the populations for all seven loci (Raymond and Rousset 1995). Pairwise tests for population differentiation (F_{st}) were conducted. A Mantel test was performed to detect isolation by distance between Mediterranean nesting areas. All this statistical analysis were conducted using Genepop 3.4 (Raymond and Rousset 1995). Geographic distances for the Mantel test were calculated with the aid of the program ArcView 3.1[©]. Furthermore, the programme bottleneck 1.2 (Cornuet and Luikart 1996) was used to detect recent bottlenecks in populations. N_e was estimated using the average dinucleotide repeat mutation rate of the sea turtle *Chelonia mydas* (2×10^{-3}) (Ellegren 2000) but the maximum and minimum values (9.6×10^{-3} and 5.7×10^{-4}) (FritzSimmons 1998) were also used in order to assess availability. The infinite-allele model (IAM; Kimura and Crow 1964) predicts $H = 4N_e\mu/(1 + 4N_e\mu)$ while the stepwise mutation model (SMM; Ohta and Kimura 1973) predicts $H = 1 - (1/\sqrt{1 + 8N_e\mu})$. The number of migrants between each population pair (N_m) was calculated from the F_{st} values using the formula

$$N_m = \frac{1}{4} \left(\frac{1}{F_{st}} - 1 \right) \text{ (FitzSimmons et al. 1997b).}$$

Results

Genetic structure of nesting sites in the eastern Mediterranean

As much as five haplotypes were found in the considered nesting areas. Haplotype CC-A6 had been reported previously only from Mediterranean nesting beaches (Encalada *et al.* 1998), whereas haplotypes CC-A2 and CC-A3 are known to be shared by Mediterranean and Atlantic nesting beaches (Encalada *et al.* 1998, Laurent *et al.* 1998). Haplotypes CC-A29 and CC-A32 had not been reported previously from any nesting beach but are present in western Mediterranean feeding grounds.

Both the new and the previous data sets from Cyprus exhibited only haplotype CC-A2. The situation with the two available Turkish data sets was completely different. Although they shared the same haplotypes (CC-A2 and CC-A3), statistically significant differences in the frequencies were found either by the Chi-square test or the Zs* test ($p < 0.05$). Furthermore, both data sets had been collected from two different and distant areas and hence they were considered to be independent and treated as different units for further analysis.

Although all the considered populations shared the haplotype CC-A2, pair-wise genetic distances revealed the existence of highly significant differences between some of the considered nesting sites ($\text{Gamma}_{st} = 0.183$, $p < 0.001$, Table 1A for pairwise comparisons), although the overall pattern was puzzling. The nesting sites of Zakynthos, Lakonikos and Kyparissia exhibited haplotypes CC-A2 and CC-A6 with very similar relative frequencies (Tables 2 and 3A) and were grouped as Greece (GRE). Furthermore, the nesting sites of Cyprus and Crete exhibit only the haplotype CC-A2 and were grouped as Islands (ISL). Although CC-A2 was the only haplotype found in Lebanon, that area was not clustered with Cyprus and Crete because sampling size was small and hence other haplotype might have not been detected by chance. Once grouped, almost all pairwise comparisons were significant and genetic distances were usually high (Table 1B). Thus, grouped populations were considered units as defined in terms of mtDNA.

Genetic diversity was very different among populations either in terms of haplotype diversity (H) or nucleotide diversity (π) (Table 2). Effective population

size (N_e) was very low for all populations. Estimates of gene flow (N_m) for mtDNA were highly variable (Table 3).

All the microsatellite loci were polymorphic, with an average of 10.43 (Table 6). However, some microsatellites (cc141, ccar176 and Ei8) usually did not amplify with some samples, probably due to the status of dead embryos in the nest. Thus, the actual sample size for this three markers in some populations was lower than that expected. Amplification problems were dramatic for the locus Ei8 in Zakynthos (Table 6).

No departure from the Hardy-Weinberg equilibrium (Chi square, $p > 0.05$) was detected for any population, although they did for some loci. Furthermore, no linkage disequilibrium was found between all loci pairs and hence, independence of loci could be assumed (Chi square, $p > 0.05$). Mean expected heterozygosity per location ranged from $H_e = 0.051$ (Zakynthos) to $H_e = 0.74$ (Lebanon). However, mean values were not very different among populations. Observed heterozygosity per locus was highly variable, ranging from $H_o = 0$ (cm72 in Zakynthos) to $H_o = 0.9$ (cc7 in Lakonikos). Significant genetic structure was observed ($F_{st} = 0.006$, $p < 0.001$). However, differences were not statistically significant for more than half the pairwise comparisons (12 of 21). Furthermore, statistical significance often involved distant nesting areas. F_{st} values were usually very low (Table 1A). The same grouping as mtDNA was done but results didn't change substantially (Table 1B). Groups still did not depart from the Hardy-Weinberg equilibrium (Chi square, $p > 0.05$).

Genetic differences could not be explained by isolation by distance, as Mantel test was not significant either after or before grouping nesting sites (Mantel test, $p > 0.05$). Moreover, no bottleneck effects were detected for any population (Wilcoxon test $p > 0.05$). Effective population size values (N_e) were not very different among populations, despite the great differences in the estimated population size (Table 4). Gene flow (N_m) between populations was highly variable (Table 5) but in any case lower than 15 individuals per generation. However nDNA estimates of gene flow were not only much higher than those calculated for mtDNA ones (paired t-test $p > 0.05$), but were statistically significant only when populations that differing in mtDNA, but not in nDNA (Table 5), were included (paired t-test $p < 0.01$).

The genetic structure of feeding grounds

A total of 15 distinct haplotypes were detected in the present study, six of them not been described previously (CC-A27 to CC-A32). However, these new haplotypes were rare, with frequencies lower than 5%. Haplotypes reported previously from Atlantic rookeries only (CC-A1, CC-A5, CC-A7, CC-A9, CC-A10, CC-A13 and CC-A14) were found in 53 individuals and hence these turtles were considered to have an Atlantic origin. The endemic Mediterranean haplotype CC-A29 was found off NE Spain, although it was found only in Israeli nesting beaches. Most of the analyzed turtles (216 individuals) had one of the two haplotypes shared by Atlantic and eastern Mediterranean rookeries (CC-A2 and CC-A3) and, therefore, could not be assigned to any nesting population. Finally, 11 turtles had haplotypes that were not found at any nesting site, indicating that data on the nesting populations whose juveniles exploit the western Mediterranean are still incomplete.

The genetic composition of the turtles in the eight foraging grounds was not homogenous (Chi-square and Snn tests; Table 4) and differences still remained significant between western Mediterranean grounds (Chi-square and Snn; Table 4) after the removal of the Atlantic feeding grounds, thereby demonstrating strong genetic structuring within the Mediterranean basin.

Table 1: Partitions among Mediterranean nesting populations. Above the diagonal are shown the F_{st} values for nDNA, below the diagonal are partitions based on mtDNA (Γ_{st} values). Table (A) nesting populations before grouping (abbreviations are defined in Table 2). Table (B) nesting populations after grouping as follows GRE (ZAK, KYP and LAK), ISL (CRE and CYP). Black cells in table B highlight pairwise comparisons involving populations with low sample size (WTU and LEB) that might produce spurious results for mtDNA (see text). (-) means that no nDNA data is available for this dataset.

A	ZAK	KYP	LAK	CRE	WTU	CYP	ETU	LEB	ISR
ZAK		-	0,005	0,003	0,022*	0,012*	-	0.019*	0.017*
KYP	0.034		-	-	-	-	-	-	-
LAK	0.034	0.007		0.001	0,002	-0,006	-	0.002	0.013*
CRE	0.046	0.048	0.027		0.007*	0.006	-	-0.004	0.005
WTU	0.022	0.041	0.030	0.035		0.004	-	0.008*	0.014*
CYP	*0.064*	*0.067	0.038	0.000	0.047		-	-0.001	0.011*
ETU	**0.096*	**0.188**	**0.188***	**0.203**	*0.127*	***0.286***		-	-
LEB	0.030	0.031	0.018	0.000	0.023	0.000	*0.131*		0.008
ISR	0.061	0.073	0.070	0.086	0.067	**0.116*	**0.181**	0.057	

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (for mtDNA right Chi-square test, left Z_s^* test)

B	GRE	ISL	WTU	ETU	LEB	ISR
GRE		-0.016	0.007*	-	0.009**	0.013**
ISL	**0.031**		0.002*	-	-0.017	-0.003
WTU	0.009	*0.054		-	0.008*	0.014*
ETU	***0.165***	***0.336***	*0.127*		-	-
LEB	0.012	0.000	0.023	*0.131*		0.008
ISR	**0.043	***0.234**	0.067	**0.181**	0.057	

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (for mtDNA right Chi-square test, left Z_s^* test)

Table 2: Comparison of effective population size (Ne) among populations as suggested by different markers. Values of Ne in brackets show the values obtained from the higher and lower values of dinucleotide mutation rates reported on the bibliography. Haplotype diversity (H), nucleotidic diversity (π) and gene diversity (He) are shown for each population. Population size is estimated through mean annual nesting values found in Margaritoulis *et al.* 2003 and assuming equal sex ratio and a mean two years reneesting interval. In brackets are shown the maximum and minimum registered values. (-) means that no nDNA data is available for this dataset.

	mtDNA			nDNA			Annual nesting females (Margaritoulis <i>et al.</i> 2003)	Estimated population size
	H	π	Ne	He	Ne IAM	Ne SMM		
ZAK	0,458	$1,21 \times 10^{-3}$	23	0,51	131 (27-458)	199 (41-698)	1301 (857-2018)	5205 (3428-8072)
KYP	0,181	$4,8 \times 10^{-4}$	9	-	-	-	581 (286-927)	2323 (1144-3708)
LAK	0,105	$2,8 \times 10^{-4}$	5	0,63	209 (44-734)	384 (80-1348)	192 (107-239)	768 (428-956)
CRE	0	0	0	0,66	247 (51-867)	491 (102-1723)	387 (315-516)	1549 (1260-2064)
WTU	0,125	$3,3 \times 10^{-4}$	6	0,65	236 (49-829)	460 (96-1613)	124 (88-158)	496 (352-632)
ETU	0,498	$1,31 \times 10^{-3}$	25	-	-	-	$\cong 100$	$\cong 400$
CYP	0	0	0	0,70	297 (62-1043)	651 (136-2284)	572 (404-775)	2286 (1616-3100)
LEB	0	0	0	0,74	360 (75-1261)	876 (183-3075)	Sporadic	Very low
ISR	0,281	$7,4 \times 10^{-4}$	14	0,69	284 (59-995)	605 (126-2123)	33 (10-52)	131 (40-208)

Table 3: Migration estimates (Nm) between Mediterranean populations based either on mtDNA or nDNA genetic distances. Significance of pair-wise comparisons is also shown. Shaded rows highlight the two pairwise comparisons that are different in terms of mtDNA but not in terms of nDNA. Black cells highlight pairwise comparisons involving populations with low sample size (WTU and LEB) that might produce spurious results for mtDNA (see text).

Population pair	Nm (mtDNA)	Differences (mtDNA)	Nm (nDNA)	Differences (nDNA)
GRE-WTU	5,06	No	35,46	Yes*
GRE-ISL	15,63	**Yes**	>1000	No
GRE-ETU	2,53	***Yes***	-	-
GRE-ISR	11,13	**Yes	18,98	Yes**
GRE-LEB	41,17	No	27,53	Yes**
WTU-ISL	8,76	*Yes	124,75	Yes*
WTU-ETU	3,44	*Yes*	-	-
WTU-ISR	6,96	No	17,61	Yes*
WTU-LEB	21,24	No	31,00	Yes*
ISL-ETU	0,99	***Yes***	-	-
ISL-ISR	1,64	***Yes**	>1000	No
ISL-LEB	>1000	No	>1000	No
ETU-ISR	2,26	**Yes**	-	-
ETU-LEB	3,32	*Yes*	-	-
ISR-LEB	8,27	No	31,00	No

*p<0.05; **p<0.01; ***p<0.001 (for mtDNA right Chi-square test, left Zs* test)

Pair-wise comparison of all foraging grounds, either by the Neighbour Joining tree (Figure 4) or by the Chi-square and Snn tests (Table 4), revealed two clusters. One included the foraging grounds off Madeira, the Azores and the Gimnesies Islands, and the other those located off the shores of the Italian and the north-eastern Spain. The members of the former cluster exhibited a higher prevalence of the haplotypes reported for Atlantic rookeries only (mainly CC-

A1), while turtles in the latter group showed a higher prevalence of CC-A2, known from the two main nesting areas, but occurring at higher frequencies in Mediterranean rookeries. Lampedusa and the Pitiüses islands were in an intermediate position in relation to the feeding grounds of the two clusters, indicating a cline in the frequency of these two haplotypes (Figure 5).

Figure 4: Neighbour-joining unrooted tree showing the genetic relationships between the feeding grounds and clusters defined in Table 8.

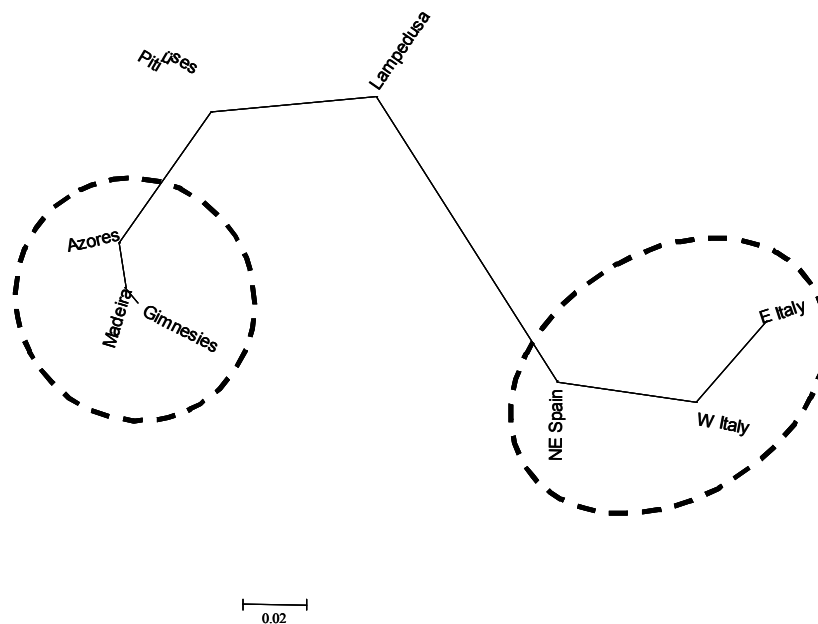
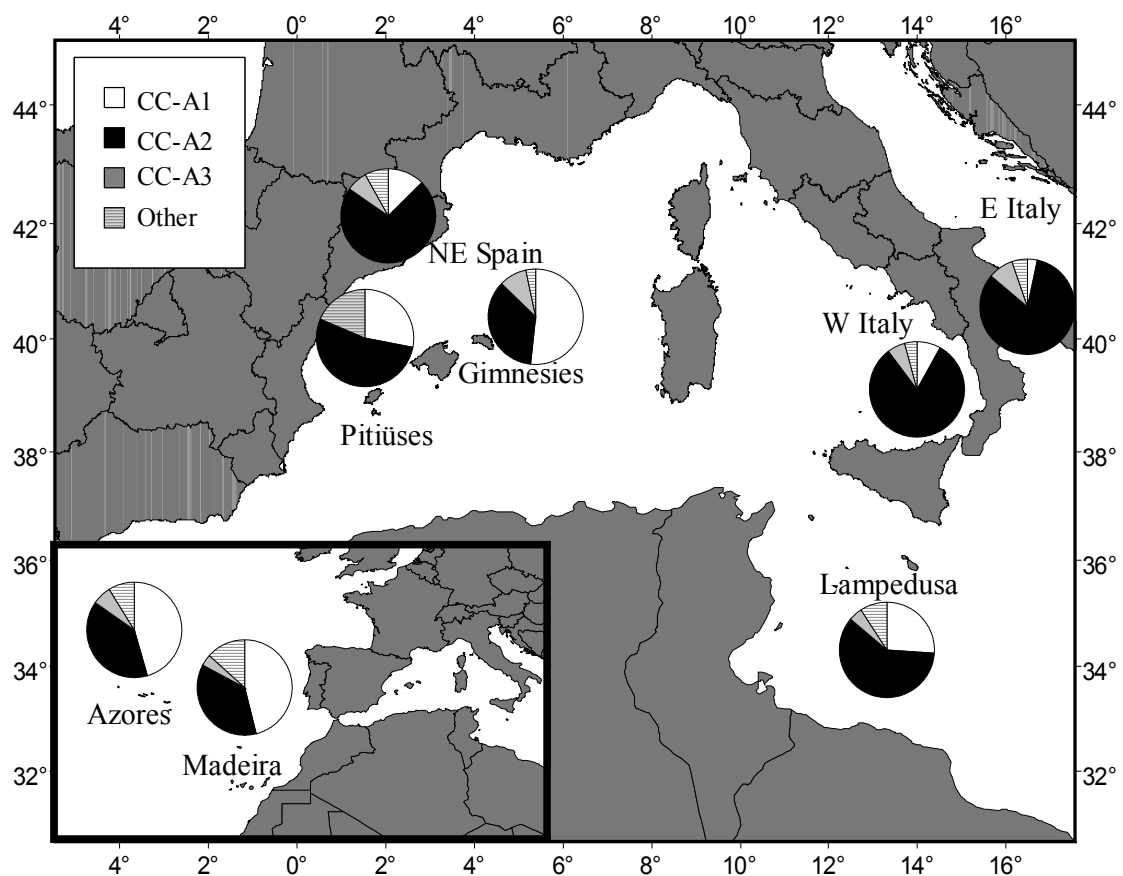


Table 4: Pair-wise genetic distance (Γ_{st}) between foraging grounds.

^aChi-square test ($p < 0.05$). ^b S_{nn} test ($p < 0.05$). Shaded cells show two homogeneous clusters. Black cells highlight a statistically significant difference between members.

	Gimnesi				Lamped	NE	
	es	Azores	Madeira	Pitiüses	usa	Spain	W Italy
Azores	0.001						
Madeira	0.003	0.001					
Pitiüses	0.041 ^{ab}	0.027	0.017				
Lampedus							
a	0.067 ^b	0.059 ^a	0.170 ^{ab}	0.006			
NE Spain	0.168 ^{ab}	0.167 ^{ab}	0.151 ^{ab}	0.055 ^{ab}	0.032		
W Italy	0.256 ^{ab}	0.215 ^{ab}	0.170 ^{ab}	0.108 ^{ab}	0.056	0.004	
E Italy	0.340 ^{ab}	0.287 ^{ab}	0.231 ^{ab}	0.173 ^{ab}	0.101 ^{ab}	0.022	0.010

Figure 5: Haplotype frequencies in each feeding ground. Haplotypes other than CC-A1, CC-A2 or CC-A3 were grouped due to its low frequency.



Discussion and conclusions

The genetic structure of the loggerhead sea turtles rookeries

Dutton (1995) recommended a sample size of 15-30 individuals for marine turtle studies using mtDNA. Sampling protocols for the present study followed this recommendation, although in some areas it was impossible due to the reasons above reported. The control region of the D-loop is highly variable in the loggerhead sea turtle but in the Mediterranean populations it is much less variable than in the Atlantic ones (Encalada *et al.* 1998, Pearce 2001). Moreover, one haplotype is widespread and found at very high frequency at all nesting beaches (mean 90%). For these reasons, the recommended sample size was high enough to allow the detection of rare haplotypes, but was too small for revealing the genetic structuring of the considered rookeries. Such a shortcoming was overcome clustering those populations that share haplotypes with similar frequencies, in order to increase sample size.

The existence of exclusive haplotypes leads to the existence of, at least, three well defined independent management units within the eastern Mediterranean, each one characterised by one exclusive haplotype: (1) CC-A6 for the nesting beaches located on mainland Greece and adjoining islands (Zakynthos, Lakonikos and Kyparissia), (2) CC-A3 for eastern Turkey and (3) CC-A29 for Israel. Cyprus and Crete are different from these three groups, but concluding that they form a unit on the basis of the sole existence of the widespread CC-A2 haplotype is not sound. Perhaps they are, but without further evidence is hard to say whether they are two independent management unit or not. The shortest geographic distance between these four or five mtDNA defined units is about 200 km (from Crete to mainland Greece), which agrees with the minimum distances suggested by other authors to originate mtDNA structure based on females philopatry in discontinuous nesting habitats (Schroth *et al.* 1996, Pearce 2001) and previously suggested also by tag recapture data (Richardson 1982).

Genetic structuring was also found when nDNA markers were considered, both using the original sampling units and those resulting from

clustering in agreement with mtDNA. nDNA data didn't revealed differences between those nesting sites that had been clustered on the basis of mtDNA data, thus supporting such grouping. Conversely, nesting sites differing in terms of mtDNA did not differ when nDNA is considered, e.g. the Islands compared with Greece or Israel. Moreover, genetic distances between populations were generally low and similar to those previously found between north Atlantic nesting beaches (Pearce 2001). Finally, nDNA gene flow is significantly higher than that suggested by mtDNA. All these evidences demonstrate male-mediated gene flow between different mtDNA defined populations and suggest that philopatry in males is less stringent than in females. Cyprus and Crete play a major role in this male-mediated gene flow, as these two islands are not different from the surrounding populations when the nDNA is considered, with the exception of western Turkey. Probably the islands act as a shortcut due to its central geographical position and prevent isolation by distance between coastal populations. However, nDNA markers still reveal genetic structuring, a result implying that males exhibit some degree of phylopatry, although at a coarser geographic scale than females. A similar conclusion had already been reported for the loggerhead sea turtle (Schroth *et al.* 1996) and for the green turtle (FitzSimmons *et al.* 1997a 1997b, Roberts *et al.* 2004).

The origin of the loggerhead sea turtles in Mediterranean feeding grounds

Previous findings indicated that loggerhead sea turtles found off the Azores and Madeira are from the western Atlantic nesting sites (Bolten *et al.* 1998), while those in the western Mediterranean originate both in the Atlantic and eastern Mediterranean nesting beaches (Laurent *et al.* 1993, Laurent *et al.* 1998, Casale *et al.* 2002). Those preliminary studies assumed a homogenous admixture of loggerhead sea turtles from the two nesting areas within the western Mediterranean, a hypothesis not supported in the present work by the existence of statistically significant differences in the genetic structure of several western Mediterranean feeding grounds. Furthermore, the Gimnesies islands clustered with the Azores and Madeira, and not with Pitiüses islands, the north-

eastern Spain and the Italian feeding grounds as might be expected if the western Mediterranean was genetically homogenous.

Processes as genetic drift or isolation by distance cannot be invoked to explain the above reported genetic structuring, as there is no reproduction in the foraging grounds and hence these sampling units cannot be treated as populations. Such differences in haplotype composition within the western Mediterranean must be generated by phenomena affecting the mixing of Atlantic and eastern Mediterranean juveniles after they reach the foraging grounds through the Strait of Gibraltar and the Strait of Messina and the Sicilian channel. This mixing do not affect to the breeding populations since the considered juveniles spend no more than a few years in the western Mediterranean before coming back to their natal beaches for breeding (Bolten 2003).

Such a distribution cannot be explained by a simple diffusion model assuming that Atlantic turtles spread into the western Mediterranean from the Strait of Gibraltar and that eastern Mediterranean turtles spread into the basin from the Sicilian channel and the Strait of Messina, as there is no significant correlation between genetic distance and geographic distance. Conversely, the pattern of surface sea currents explains successfully the reported genetic heterogeneity.

Highly saline water from the Tyrrhenian Sea flows along the western coast of Italy until it reaches the Ligurian Sea (Millot 1987, Millot 2001, Pinardi and Masetti 2000). There, the Liguro-Provençal current originates and flows westward, carrying water from the Ionian Sea to the south-eastern Iberian Peninsula. At Cape la Nao, a branch of this current is deflected towards the south-western Balearic Islands (the Pitiüses) and supplies these islands with Mediterranean water (Millot 1987, Millot 2001, Pinardi and Masetti 2000). The percentage of turtles with the haplotype CC-A2 significantly decreases downstream along the Liguro-Provençal current, although there is no correlation when the geographic distance is used, hence demonstrating that turtles disperse mainly along the Liguro-Provençal current. Actually, the Liguro-Provençal current is a short-cut that accelerates the transport of eastern Mediterranean turtles along the European shore to the western Mediterranean.

The Algerian current flows eastward from the Strait of Gibraltar to the Sicilian channel (Millot 1987, Millot 2001, Pinardi and Masetti 2000), thus transporting Atlantic water with a low salinity along the African coast. Temporary eddies leave the Algerian current and supply the Balearic Islands (Encalada *et al.* 1998) and Sardinia with Atlantic water. However, the relative abundance of the haplotype CC-A1 at the foraging grounds influenced by this current does not decrease significantly eastward. This is because the Liguro-Provençal current has a stronger effect on the Pitiüses islands (SW Balearic Islands) than on the Gimnesies islands (NE Balearic Islands) (López-Jurado *et al.* 1990) and no effect at all on Lampedusa (Malanotte-Rizzoli *et al.* 1997). As a consequence, the supply of eastern Mediterranean turtles carrying the CC-A2 haplotype and the resulting “dilution” of the CC-A1 haplotype is unrelated to the distance to the source of Atlantic turtles.

As a result of this current pattern, and consistent with a scenario in which immature loggerhead sea turtles remain linked to their original water masses, the foraging grounds off the north African coast and the north-eastern Balearic archipelago (the Gimnesies Islands) would be inhabited mainly by turtles of the Atlantic stocks, as these regions are not affected by currents from the eastern Mediterranean. Conversely, the foraging grounds off the European shore of the western basin, and those in the eastern basin, would be mainly inhabited by turtles from the eastern Mediterranean rookeries, as little Atlantic water reaches these regions (Millot 1987, López Jurado 1990). Finally, the foraging grounds located in areas receiving both Atlantic and eastern Mediterranean water (Lampedusa islands and the Pitiüses islands) would be inhabited by specimens of both origins. The reason why immature loggerhead sea turtles remain linked to their original water masses remains unknown.

Finally, although previous mtDNA studies suggested that juveniles born in the eastern nesting sites exploit the feeding grounds in the western Mediterranean, on the basis of the high frequency of occurrence of the common CC-A2 haplotype (Encalada *et al.* 1998, Laurent *et al.* 1993, Laurent *et al.* 1998), none of the exclusive haplotypes of Greece and Israel had been found. The present study reports their existence for the first time in the western Mediterranean, thus providing hard evidence that eastern Mediterranean turtles overlap with Atlantic turtles in the western Mediterranean. The presence of the

Israeli haplotype is striking, considering the small size of the population and hence the small number of offspring produced every year.

Conservation implications

The complex population structuring observed in the eastern Mediterranean rookeries and the pelagic foraging grounds have profound conservation implications that should be considered in any management plan.

A coarse analysis of the available information would conclude the existence of two groups of populations that may be genetically depressed and hence collapse on the short run. The first group involves the extremely small populations of Lebanon and Israel (Margaritoulis *et al.* 2003). The second one includes the insular populations of Crete and Cyprus, where only the ancestral haplotype CC-A2 is found. Such a low genetic variability has also been reported from the western Atlantic, where the northern populations exhibit only the haplotype CC-A1 (Encalada *et al.* 1998). Strikingly, all those populations exhibit a high nuclear variability, similar to that of other Mediterranean rookeries. Moreover, nDNA did not reveal any recent bottleneck for any of them. Finally, the reduced populations of Israel and Lebanon have effective nuclear population sizes (N_e) higher than their real population size, something biologically impossible unless they are linked to other populations by an intense male-mediated gene flow. The final conclusion is that none of those populations is genetically isolated and that long-term viability is not hindered by inbreeding and genetic depression.

A second major conclusion deals with population expendability. Genetics is a powerful tool that provides highly valuable information for managing threatened species. However, genetic data have to be carefully analysed, as biases may lead to wrong decision with negative consequences on the considered species. The loggerhead turtle in the Mediterranean provides a nice example of these shortcomings, as nDNA and mtDNA analysis taken alone may mandate contradictory management strategies for the conservation of the species. mtDNA information may suggest (1) that the protection of those nesting beaches with exclusive haplotypes has a priority over the protection of rookeries without exclusive haplotypes and (2) that the conservation of

extremely small populations is probably useless because their survival is hindered due to inbreeding. On the other hand, nDNA data may suggest that a lower number of management units should be even considered for conservation purposes. However, the combination of both sources of information reveals the existence of a much complex population structure that cannot be preserved if only a few rookeries are preserved.

The central role of Cyprus and Crete is revealed only by a fine analysis of mtDNA and nDNA data. However, the application of genetic data to biodiversity conservation is often much coarser and populations without private alleles or haplotypes are thought to deserve a lower attention (Ruckelshaus *et al.* 2003). That approach would suggest that the nesting sites of Cyprus and Crete are not so important as those in mainland Greece and Turkey, as they contain only a widespread mtDNA haplotype and share almost all nDNA alleles with other sites (Appendix I). Indeed, these two islands probably link the populations in the Levant (Israel and Lebanon) with those from Greece thus allowing gene flow between all Mediterranean nesting beaches. As a consequence, viability of small populations on the long run might depend on the conservation of the insular populations.

However, the protection of nesting sites will be useless if mortality at the foraging grounds is not reduced. Every year, several thousand immature individuals are caught accidentally by a variety of fishing activities in the western basin (Aguilar *et al.* 1995; Carreras *et al.* 2004) and in the eastern basin (Godley *et al.* 1998; Casale *et al.* 2004). This mortality poses a threat both to the Atlantic and the eastern Mediterranean populations (Margaritoulis *et al.* 2003, NMFS-SEFSC 2001) as juveniles from both nesting areas use foraging grounds in the western Mediterranean. Since female migration between beaches is extremely rare (Schroth *et al.* 1996), the impact of mortality on the nesting stocks can vary stochastically depending on their size (Broderick *et al.* 2002). The stock of turtles nesting in the eastern Mediterranean (Margaritoulis *et al.* 2003) is much more endangered than that in the Atlantic rookeries (Ehrhart *et al.* 2003). Given that immature turtles from the former region are found throughout the eastern Mediterranean and the feeding grounds off the European shore of the western Mediterranean, a reduction in turtle by-catch and mortality in these areas should be considered a priority to protect the eastern

Mediterranean population. Conversely, specimens with haplotypes exclusive to some small Mexican populations have been found in the Algerian basin and thus by-catch and mortality there may be relevant for the conservation of those populations.

Management recommendations

1. All the nesting rookeries in the eastern Mediterranean should be preserved. This is particularly critical for the populations of Crete and Cyprus, as they form the link between the Levantine and Ionian rookeries.
2. Efforts to reduce incidental catch of loggerhead sea turtles should focus on the eastern Mediterranean, the Tyrrhenian sea, the Ligurian sea, the Gulf of Lions and the Balearic sea because these areas are used as foraging grounds for young loggerhead sea turtles from the Mediterranean nesting sites. Thus, the catch has a direct and negative impact on the highly threatened Mediterranean population.
3. The incidental catch of loggerhead sea turtles in the Algerian basin and the Alboran sea should also be reduced, although this is not so critical as in the previous case because most individuals in these areas originate from the Caribbean population, which is comparatively much less threatened. However, reducing by-catch there will also have a marginal – through positive- effect on the Mediterranean rookeries.

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