

Carlos Carreras · Sara Pont · Fulvio Maffucci
Marta Pascual · Anna Barceló · Flegra Bentivegna
Luis Cardona · Ferràn Alegre · Manuel SanFélix
Gloria Fernández · Alex Aguilar

Genetic structuring of immature loggerhead sea turtles (*Caretta caretta*) in the Mediterranean Sea reflects water circulation patterns

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Abstract The analysis of mitochondrial DNA in loggerhead sea turtles (*Caretta caretta*) from eight foraging grounds in the Mediterranean and the adjoining Atlantic revealed deep genetic structuring within the western Mediterranean. As a consequence, the foraging grounds off the North-African coast and the Gimnesies Islands are shown to be inhabited mainly by turtles of the Atlantic stocks, whereas the foraging grounds off the European shore of the western Mediterranean are shown to be inhabited mainly by turtles from the

eastern Mediterranean rookeries. 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 north of the western Mediterranean comprises mostly 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.

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C. Carreras · L. Cardona (✉) · A. Aguilar
Department of Animal Biology, Faculty of Biology,
University of Barcelona, Avda. Diagonal 645,
E-08028, Barcelona, Spain
E-mail: luis.cardona@ub.edu
Tel.: +34-934021453
Fax: +34-934034426

S. Pont · F. Alegre
Marine Animal Rescue Center (CRAM),
Camí Ral 239, E-08330, Premià de Mar, Spain

F. Maffucci · F. Bentivegna
Stazione Zoologica "A. Dorhn",
Villa Comunale I, 80121 Naples, Italy

M. Pascual
Department of Genetics, Faculty of Biology,
University of Barcelona, Avda. Diagonal 645,
E-08028, Barcelona, Spain

A. Barceló
Department of Biochemistry and Molecular Biology,
Faculty of Veterinary Science, Barcelona Autonomous University,
E-08193, Bellaterra, Spain

M. SanFélix
Marine Biology Laboratory, Department of Zoology,
Faculty of Biology, University of Valencia,
C/Dr. Moliner 50, E-46100, Burjasot (Valencia), Spain

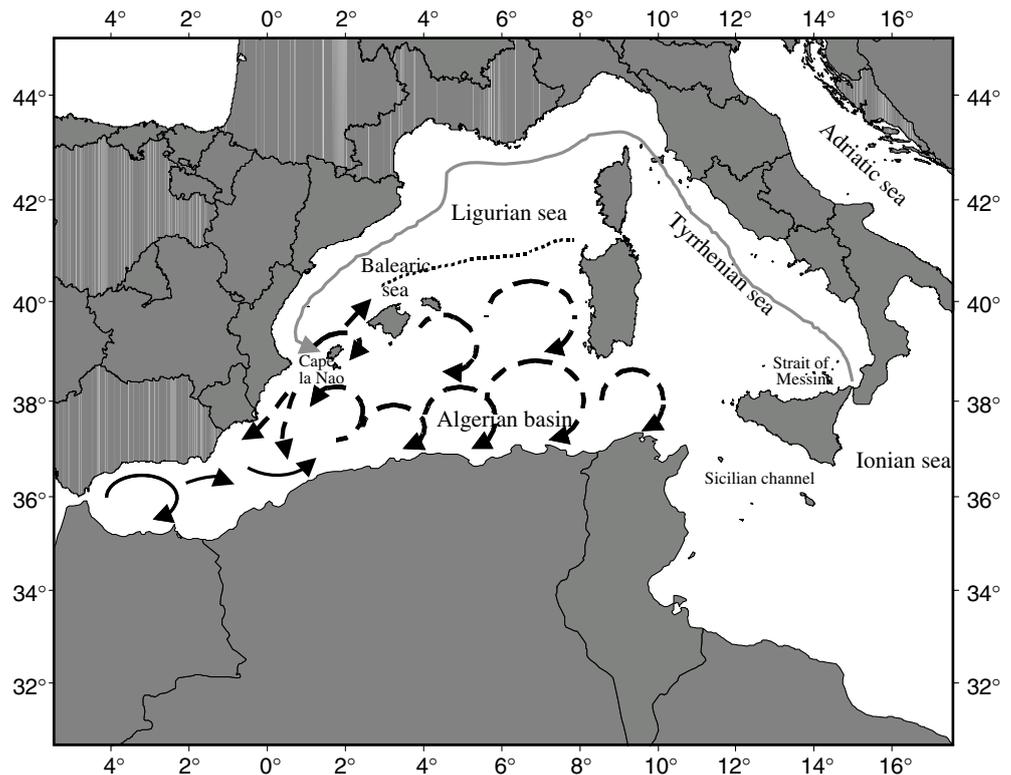
G. Fernández
Fundación Marineland, C/ Garcilaso de la Vega 9,
E-07181, Costa d'en Blanes, Calvià, Spain

Introduction

After hatching, the juveniles of most species of sea turtles spend several years offshore, dispersing over extremely large areas (Bowen and Karl 1997). The loggerhead sea turtle (*Caretta caretta*) is the most common turtle in the Mediterranean Sea (Broderick et al. 2002). The specimens inhabiting the western Mediterranean come from the western Atlantic and the eastern Mediterranean nesting areas, as nesting sites do not exist in the western Mediterranean (Laurent et al. 1993, 1998; 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. Once in the western Mediterranean, little is known about how eastern Mediterranean and Atlantic juvenile turtles mix before returning to their beaches of origin for breeding (Laurent et al. 1998).

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 (Fig. 1). The Algerian basin is filled with low-salinity water that entered recently from the Atlantic and flows eastwards along the

Fig. 1 Major current systems within the western Mediterranean in agreement with Millot et al. (1987). *Black arrows* indicate the routes followed by the Atlantic water (Algerian current) and *grey arrows* the routes of the Eastern Mediterranean water (Liguro-Provençal current). *Dashed lines* indicate temporal eddies and the *dotted line* a permanent front between the two systems



North African coast (Millot 1987, 2001; Pinardi and Masetti 2000). Conversely, the Liguro-Provençal current flows cyclonically along the European shore, thus filling the Tyrrhenian Sea, the Ligurian Sea and the Balearic Sea with saltier Mediterranean water (Millot 1987, 2001; Pinardi and Masetti 2000). This heterogeneity is known to affect the distribution of plankton, as the abundance of Atlantic species decreases counter-clockwise within the western basin (Estrada et al. 1985). However, the possible effect of major surface currents has not been considered in previous stock analyses of young loggerhead sea turtles in the Mediterranean (Laurent et al. 1993, 1998).

As loggerhead sea turtles of the Atlantic and the Mediterranean stocks gain access to the western Mediterranean using two different routes, and current systems are thought to play a relevant role in the dispersal of immature loggerhead sea turtles (Davenport and Clough 1986; Bolten 2003), a heterogeneous distribution of both type of loggerhead sea turtles within the western Mediterranean is expected, in agreement with the existence of two major water masses and current systems. Satellite tracking has revealed that loggerhead sea turtles in the Tyrrhenian Sea migrate to the eastern Mediterranean (Bentivegna 2002) but seldom enter the adjoining Algerian basin, thus avoiding Atlantic water. Conversely, loggerhead sea turtles in the Algerian basin sometimes enter the southern and eastern parts of the adjoining Balearic Sea, filled with Mediterranean water, but not the Tyrrhenian Sea (Cardona et al. 2005). Thus, exchange between sub-basins seems to be limited on the

short run. Unfortunately, satellite tags allow turtle tracking only for a few months. Here we use genetic markers to estimate loggerhead sea turtle dispersal on the long run within the western Mediterranean.

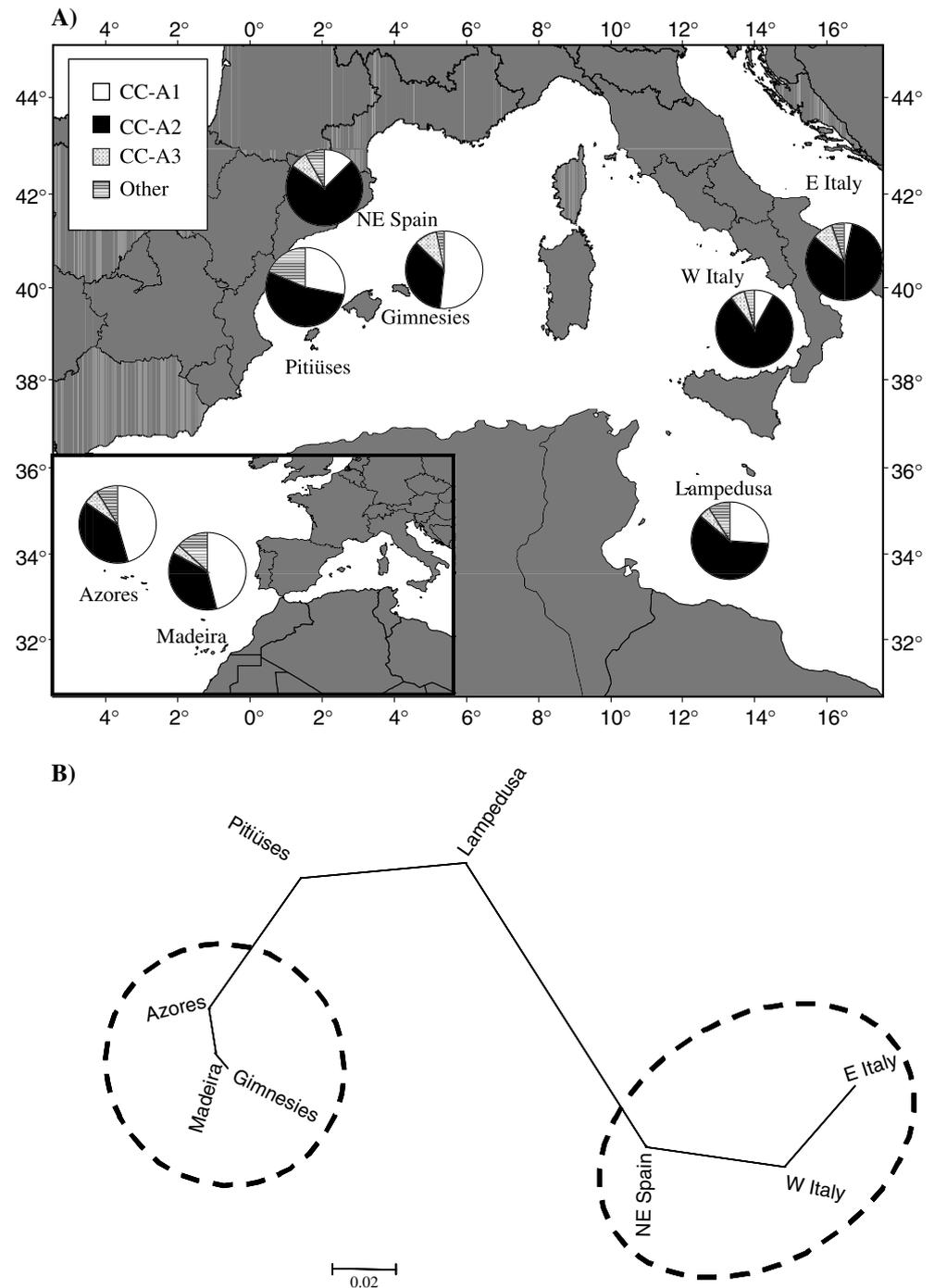
Material and methods

Material

Published data from foraging grounds off Lampedusa (LAM: Casale et al. 2002) and the Azores and Madeira (AZO and MAD: Bolten et al. 1998) were 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. In addition to these data, blood and tissue samples from 282 juvenile loggerhead turtles were collected from five locations in the western Mediterranean: Gimnesies Islands (GIM: NE Balearic Islands), Pitiüses Islands (PIT: SW Balearic Islands), north-eastern Spain (NES: from Ebro delta to the French border), western Italy (WIT: from the Strait of Messina to Cape Circeo) and eastern Italy (EIT: from the Strait of Messina to Campomarino) (Fig. 2a, Table 1). Samples were collected from live and dead individuals during four consecutive summers (from 1999 to 2003).

Live animals were caught accidentally by fishermen or collected by scuba divers while the turtles were

Fig. 2 a Haplotype frequencies in each foraging ground. Haplotypes other than CC-A1, CC-A2 or CC-A3 were grouped due to its low frequency. **b** Neighbour-joining unrooted tree showing the genetic relationships between the foraging grounds and clusters defined in Table 1



basking (Ehrhart and Ogren 2000). All turtles were tagged with metal flipper tags or subcutaneous PIT tags before release to avoid pseudoreplication. Furthermore independency of samples can be assumed considering the size of the involved populations (Ehrhart et al. 2003; Margaritoulis et al. 2003), the high mortality of turtles before they reach the size of the sampled individuals (Crouse et al. 1987) and the long distance between the putative nesting areas and the sampled sites.

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 5 M solution or stored at -20°C . Standard straight carapace length (SCL) was measured for each turtle.

Laboratory methods

DNA was extracted from blood samples either using the QIAamp blood mini kit (QIAGEN®), following the

Table 1 Foraging grounds, absolute frequencies of haplotypes, carapace length of the turtles and references

	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	CC-A	n	SCL	References				
	1	2	3	5	7	8	9	10	11	12	13	14	15	16	17	26	27	28	29	30	31	32	(cm)		
LAM	17	39	3		1	1	1				1	1			2								65	26–55 ^a Casale et al. (2002)	
AZO	36	31	5		1					2	2	2	1	1										79	7–66 ^a Bolten et al. (1998)
MAD	24	19	2			3	1	1	1	1	1	1			1									52	18–57 ^a Bolten et al. (1998)
GIM	16	11	3												1									31	50.0 ^a (9.6) 32–73 Present study
PIT	9	17	1		1		1		1	2	1	2			1									32	49.6 ^a (9.0) 28–66 Present study
NES	14	81	8	1	1			1							2		1	2	1					112	44.1 ^a (14.9) 20–100 Present study
WIT	4	40	3												1		1							49	49.7 ^a (13.7) 25–72 Present study
EIT	2	48	5												1									58	36.8 ^b (12.5) 76–65 Present study
Total	122	286	29	2	2	1	2	4	1	1	4	6	1	1	1	7	1	1	2	1	1	1	2	478	

Length information is given as mean (bold), range (italics) and standard deviation (brackets) of the straight carapace length (SCL) in centimetres

Average length values with different superscript are statistically different (see text for details)

^aData originally collected as curved carapace length (CCL) and transformed to SCL using the equation $CCL = 1.388 + (1.053) SCL$ (Bjørndal et al. 2000)

manufacturer's instructions, or by proteinase K/SDS digestion and ethanol precipitation. DNA was extracted from tissue either using the QIAamp extraction kit (QIAGEN®) or by incubating a small piece of tissue (0.5–1 g) for 3 h at 56°C with 1 ml of 6 M Guanidine chloride (Sigma) followed by a purification using Wizard DNA Clean-Up System (Promega).

A fragment of the D-loop of the mitochondrial control region 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). The PCR protocol was 94°C for 5 min, followed by 30–35 cycles at 94° for 1 min, 55°C for 1 min and 72°C for 1 min with a final extension at 72°C for 10 min. PCR products were visualized in a 1% agarose gel and purified with the QIAquick kit (QIAGEN®). Purified products were sequenced with the BigDye™ Primer Cycle Sequencing Kit (Applied Biosystems) or the DY-Enamic™ ET Terminator Cycle Sequencing Kit (Amersham) and analysed on an ABI 3700 or ABI 373 automated DNA Analyser (Applied Biosystems). New haplotypes were sequenced in both forward and reverse directions to ensure accuracy.

Data analysis

Sequences were aligned using the BioEdit programme version 5.0.9 (Hall 1999) and compared with the previously found haplotypes (Laurent et al. 1998; Bolten et al. 1998; Encalada et al. 1998), described in the Archie Carr Center of Sea Turtle Research DNA database (<http://www.acstr.ufl.edu/>). New haplotypes were assigned a code following the established nomenclature. A haplotype maximum parsimony tree was built using the Mega3 package (Kumar et al. 2001).

The nesting populations that provide turtles to each foraging grounds were assessed using the mixed stock analysis. The traditional maximum likelihood approach has been reported to be biased by the presence of rare haplotypes such as singletons or those occurring at fre-

quencies lower than 5%, a shortcoming overcome by Bayesian approaches (Pella and Masuda 2001). This is crucial in this study as the control region of the D-loop of the mtDNA in the loggerhead turtle is characterized by the presence of rare haplotypes both in the nesting populations and the foraging grounds. Hierarchical Bayesian models also allow to combine genetic and ecological data, like rookery size, to avoid the over representation of extremely small populations typical of other Bayesian approaches (Okuyama and Bolker 2004). Thus, the programme Bayes (Pella and Masuda 2001) was run in two different ways. The Bayesian model BM1 did not consider rookery size, whereas the model BM2 weighted the contribution of each rookery by the size of the nesting population. Haplotype frequencies of the Atlantic nesting sites of north-western Florida (NWFL), South Florida (SFL), north-east Florida–North Carolina (NEFL–NC), Dry Tortugas and Mexico were obtained from Encalada (1998) and Bowen (2005), while haplotypes frequencies of the Mediterranean nesting sites at Greece and Turkey were obtained from Laurent et al. (1998) (Table 2). Nesting population sizes were obtained from Margaritoulis et al. (2003) and Ehrhart et al. (2003) (Table 2). The Gelman-Rubin shrink factor was used to test for anomalous realizations of the Bayes predictive posterior distribution (Pella and Masuda 2001). Values greater than 1.20 indicated a lack of convergence in the algorithm and the corresponding estimates were considered unreliable. Analysis was also done by grouping the nesting populations in two regions (Atlantic and Mediterranean).

The impossibility of statistical comparisons between the results provided for each foraging ground is one of the major shortcomings of the mixed stock analysis when more than one mixed stocks are included. This was overcome by assessing the heterogeneity of haplotype frequencies between foraging grounds by means of the Chi-square test (Zaykin and Pudovkin 1993). Values were compared to the distributions observed by randomizing individuals among populations using Monte-Carlo resampling (Rolf and Bentzen 1989), as

Table 2 Source nesting populations used in the Bayesian mixed stock analysis

	CC-A1	CC-A2	CC-A3	CC-A4	CC-A5	CC-A6	CC-A7	CC-A8	CC-A9	CC-A10	CC-A11	CC-A14	CC-A20	Total	Rookery size
NWFL	38	7	2				2							49	600
SFL	52	45	4		1		3				1	2	1	109	67,100
NEFL-NC	104	1												105	6,200
Dry Tortugas	4	50							2	2				58	217
Mexico		11	2					1	1	5				20	1,800
Greece		78				2				1				81	3,660
Turkey		19	13											32	1,366
Brasil				11										11	2,400
Total	198	211	21	11	1	2	5	1	3	8	1	2	1		

Mediterranean data are from Laurent et al. (1998)

Atlantic data are from Encalada et al. (1998) and Bowen et al. (2005) Population sizes are obtained from Ehrhart et al. (2003) and Margaritoulis et al. (2003)

Population codes are described in the text

implemented in the CHIRXC programme (Zaykin and Pudovkin 1993). With this method, haplotypes occurring at low absolute frequencies had not to be grouped. Moreover, the Snn test (Hudson 2000) was also used because it incorporates sequence divergence information to the analysis, as implemented in DNAsp programme version 4.0 (Rozas et al. 2003). Genetic distance (Gamma_{st}) between foraging grounds was also calculated using the DNAsp, and used to reconstruct a neighbour-joining tree. A Mantel test, with GENEPOP (Raymond and Rousset 1995) was performed in order to assess whether the genetic distance between foraging grounds and the geographic distance was correlated. To test whether the Liguro-Provençal current influences the genetic structure of the foraging grounds under its influence, non-parametric correlation (Spearman Rho) was calculated between the relative abundance of haplotype CC-A2 (shared by Atlantic and Mediterranean nesting sites, but with a much higher frequency at the later) and the distance to the foraging ground found in eastern Italy. Two independent tests were conducted, one using the shortest geographic distance and the other one using the geographic distance as measured along the Liguro-Provençal current. A similar analysis was conducted to test the contribution of the Algerian current to the relative abundance of the haplotype CC-A1 (found only at Atlantic nesting sites) of those foraging grounds under its influence. Here, the shortest distance and the distance as measured along the current are almost the same and hence just one test was conducted.

One-way ANOVA, followed by a post hoc Student Newman-Keuls test, was conducted to test for the existence of differences in the average SCL of the five foraging grounds sampled in the present study. SCL data of Madeira, the Azores and Lampedusa were not included in this analysis as data were not available from the reference sources.

Results

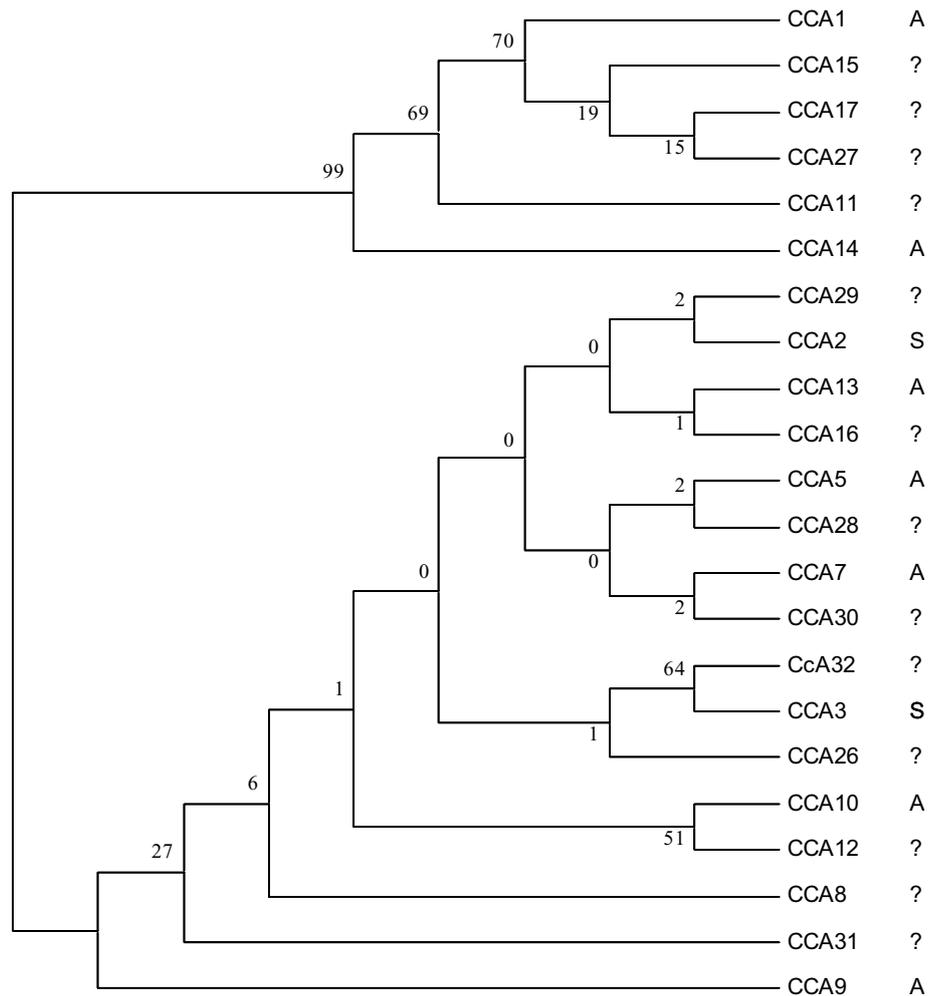
A total of 15 distinct haplotypes were detected in the present study (Table 1), six of them not described previously (CC-A27 to CC-A32, Genbank accession numbers AY742908 to AY742913). However, these new haplotypes were rare, with frequencies lower than 5%. A total of 22 haplotypes were considered in the present work after including published data from foraging grounds (Table 1). Maximum parsimony was used to reconstruct a MP tree (Fig. 3) considering all sites of the sequences, including gaps. Two distant clades with significant bootstrap values were found, with 17 fixed differences between them. 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 only endemic Mediterranean haplotype, CC-A6, described from the Greek nesting sites (Encalada et al. 1998), was not detected, a

hardly surprising result considering its low (6.4%) frequency of occurrence, even in these rookeries. Most of the analysed 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, 13 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. Indeed, only a few samples from the Libyan and Israeli rookeries have been analysed to date (Laurent et al. 1998) and there is no published information from the Cape Verde rookeries, although juveniles from all three areas may use the western Mediterranean foraging grounds.

Unweighted mixed stock analysis (BM1) suggested that the contribution of Atlantic and Mediterranean nesting sites to the turtle stock found at each of the eight considered foraging grounds was highly variable (Table 3), as most of them (Azores, Madeira, Gimnesias, Pitiúses and Lampedusa) were mainly inhabited by turtles from the Atlantic rookeries, but those along the European shore (E Italy, W Italy and NE Spain) were inhabited mainly by turtles from the Mediterranean rookeries. Most of the Atlantic turtles come from the populations of SFL, NEFL-NC and Dry Tortugas, while those born in the Mediterranean come both from Greece or Turkey (Table 3). However, the high contribution of the small population from Dry Tortugas (Table 2) to some of the considered foraging grounds (Table 3) seems unrealistic on the ground of the small size of that nesting populations. When the data were weighted in agreement with population size (BM2), the mixed stock analysis yielded a similar scenario (Table 3, Fig. 4), but the contribution of Dry Tortugas to most of the considered foraging grounds was reduced dramatically, but for Lampedusa. Conversely, the contribution of the larger SFL population increased.

The results of the Chi-square and the Snn tests also supported the existence of deep heterogeneity in the composition of the eight considered foraging grounds (Chi-square and Snn tests; $P < 0.05$). Differences still remained significant after the removal of the Atlantic foraging grounds of Azores and Madeira (Chi-square and Snn; $P < 0.05$), thereby demonstrating strong genetic structuring within the Mediterranean. Pairwise comparison of all foraging grounds, either by the neighbour-joining tree (Fig. 2b) or by the Chi-square and Snn tests (Table 4), revealed two clusters. One included the foraging grounds off Madeira, the Azores and the Gimnesias Islands, and the other those located off the shores of Italy and 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 the Mediterranean rookeries.

Fig. 3 Maximum parsimony tree of haplotypes and nesting area in which each haplotype was described (*A* Atlantic, *S* shared by the Atlantic and Mediterranean nesting areas and ? unknown nesting area). Bootstrap values are shown in each branch



The foraging grounds off Lampedusa and the Pitiüses islands were in an intermediate position (Table 4, Fig. 2), thus revealing the existence of a cline in the frequency of these two haplotypes. The CC-A1 Atlantic haplotype decreased eastwards and, as a consequence, the CC-A2 haplotype increased in the same direction (Fig. 2). However, this cline did not reflect a simple geographical trend in variability. The Gimnesies Islands, located at the centre of the western Mediterranean, clustered with the Atlantic islands of the Azores and Madeira, while the north-eastern Iberian Peninsula, roughly located at the same longitude, clustered with the two grounds off the Italian peninsula. Finally, the foraging grounds off the Pitiüses Islands, situated only 150 km south of the Gimnesies Islands, occupied an intermediate position in the tree.

As a consequence, no correlation exists between genetic and geographic distances (Mantel test; $P > 0.05$). Furthermore, there is no correlation between the relative abundance of CC-A2 at the foraging grounds influenced by the Liguro-Provençal current (eastern Italy, western Italy, north-eastern Spain, Pitiüses islands and Gimnesies islands) and the shortest distance to eastern Italy (Spearman Rho = -0.6 ; $P > 0.05$), although the corre-

lation becomes highly significant when the distance as measured along the current is used (Spearman Rho = -1.0 ; $P < 0.001$). Conversely, there is no significant correlation between the relative occurrence of the haplotype CC-A1 at the foraging grounds influenced by the Algerian current (Azores, Madeira, Pitiüses islands, Gimnesies islands and Lampedusa) and the distance to Azores (Spearman Rho = -0.3 ; $P > 0.624$).

Despite general agreement between the two methods here used (mixed stock analysis and pairwise comparison by means of the Chi-square and Snn tests), the relative contributions of Atlantic and Mediterranean nesting sites to the foraging grounds off Pitiüses and Lampedusa are controversial. On the one hand, the mixed stock analysis, either BM1 or BM2, revealed an extremely high occurrence of Atlantic turtles off Pitiüses and Lampedusa (Table 3, Fig. 4). On the other hand, none of them was completely clustered with those inhabited mainly by Atlantic turtles (Azores, Madeira and Gimnesies) (Table 4, Fig. 2b).

Finally, the mean SCL of the turtles was not homogeneous within the five Mediterranean foraging grounds sampled in the present study (ANOVA; $df = 4$; $F = 8.512$ $P < 0.001$), as turtles found off eastern Italy were

Table 3 Relative contribution of each rookery to the considered foraging grounds as revealed by two different Bayesian mixed stock analysis models

	Azores	Madeira	Gimnesies	Pitiüses	Lampedusa	NE Spain	W Italy	E Italy
NWFL	0.0661 (0.117) 0.0009 (0.009)	0.0785 (0.135) 0.0060 (0.034)	0.1238 (0.195) 0.0043 (0.031)	0.0219 (0.055) 0.0007 (0.006)	0.0592 (0.102) 0.0018 (0.016)	0.0221 (0.042) 0.0010 (0.009)	0.0303 (0.049) 0.0012 (0.011)	0.0108 (0.022) 0.0005 (0.005)
SFL	0.7407 (0.204) 0.9372 (0.074)	0.6104 (0.232) 0.8165 (0.143)	0.6451 (0.282) 0.9476 (0.099)	0.6233 (0.179) 0.8421 (0.165)	0.4636 (0.205) 0.6470 (0.162)	0.1963 (0.098) 0.3044 (0.093)	0.0394 (0.070) 0.2203 (0.113)	0.0169 (0.035) 0.0857 (0.062)
NEFL-NC	0.0540 (0.097) 0.0182 (0.047)	0.0866 (0.128) 0.0368 (0.081)	0.0784 (0.130) 0.0277 (0.071)	0.0180 (0.042) 0.0081 (0.026)	0.0167 (0.036) 0.0074 (0.025)	0.0116 (0.025) 0.0055 (0.017)	0.0225 (0.037) 0.0068 (0.020)	0.0099 (0.019) 0.0042 (0.012)
Dry tortugas	0.0175 (0.043) 0.0003 (0.004)	0.0533 (0.096) 0.0028 (0.028)	0.0196 (0.046) 0.0004 (0.006)	0.2648 (0.174) 0.0899 (0.155)	0.3130 (0.185) 0.2249 (0.204)	0.2795 (0.280) 0.0114 (0.078)	0.2730 (0.332) 0.0186 (0.113)	0.1867 (0.267) 0.0063 (0.063)
Mexico	0.0455 (0.058) 0.0336 (0.046)	0.1305 (0.094) 0.1277 (0.088)	0.0122 (0.035) 0.0024 (0.015)	0.0163 (0.037) 0.0396 (0.071)	0.0184 (0.045) 0.0167 (0.051)	0.0115 (0.031) 0.0049 (0.029)	0.0178 (0.064) 0.0059 (0.043)	0.0133 (0.042) 0.0047 (0.032)
Greece	0.0294 (0.058) 0.0054 (0.022)	0.0185 (0.044) 0.0081 (0.035)	0.0234 (0.052) 0.0056 (0.023)	0.0409 (0.081) 0.0170 (0.058)	0.0855 (0.138) 0.0941 (0.154)	0.2867 (0.255) 0.5302 (0.131)	0.4357 (0.317) 0.6441 (0.201)	0.4476 (0.299) 0.5979 (0.239)
Turkey	0.0449 (0.068) 0.0040 (0.020)	0.0192 (0.046) 0.0015 (0.011)	0.0929 (0.119) 0.0111 (0.046)	0.0101 (0.027) 0.0019 (0.014)	0.0417 (0.071) 0.0076 (0.039)	0.1910 (0.111) 0.1424 (0.11)	0.1788 (0.142) 0.1024 (0.17)	0.3121 (0.200) 0.3001 (0.23)
Brazil	0.0018 (0.005) 0.0005 (0.003)	0.0029 (0.008) 0.0006 (0.004)	0.0046 (0.012) 0.0009 (0.005)	0.0048 (0.013) 0.0007 (0.004)	0.0020 (0.005) 0.0005 (0.003)	0.0013 (0.004) 0.0003 (0.002)	0.0027 (0.008) 0.0007 (0.004)	0.0027 (0.008) 0.0005 (0.003)

In the first one (italics), rookery size was not considered, whereas in the second one (bold types) the contribution of every rookery was weighted by the size of the nesting population. Standard errors are shown in brackets.

statistically shorter than those found in the foraging grounds of the western Mediterranean (western Italy, north-eastern Spain, the Pitiüses islands and the Gimnesies islands; Table 1).

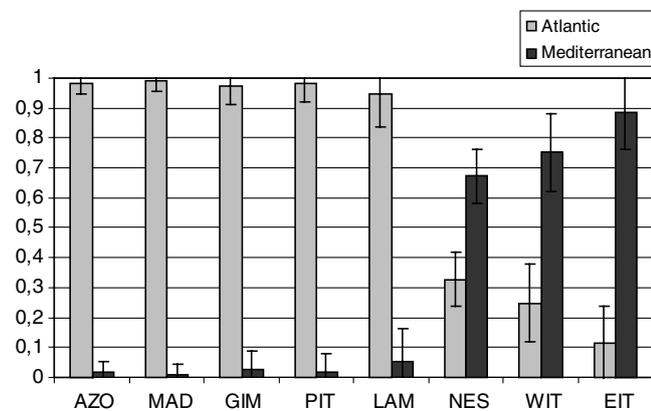


Fig. 4 Estimated contribution of Atlantic and Mediterranean nesting beaches to each foraging ground as suggested by the weighted mixed stock analysis (BM2; see text for details). Vertical bars show standard errors. Foraging grounds codes are defined in the text

Discussion and conclusions

The mixed stock analysis is a powerful technique for deciphering the contribution of several source areas to mixed stocks sharing habitats like foraging grounds. Unfortunately, it may lead unrealistic results when genetic diversity in the source populations differ greatly and small and distant sources that are just subsets of a more diverse population are included in the analysis (Engstrom et al. 2002). The Bayesian approach for mixed stock analysis try to overcome that situation, but it is still extremely sensitive to the occurrence of rare haplotypes and may also yield biased results (Bass et al. 2004), unless sources were weighted in agreement with population size (Okuyama and Bolker 2004). This is clearly demonstrated by the differences in the output of the unweighted (BM1) and weighted (BM2) mixed stock analyses of the foraging grounds here considered, as an unrealistic high contribution of the tinny population nesting at Dry Tortugas to some of the considered foraging grounds was suggested when unweighted data were used. That contribution decreased dramatically

Table 4 Pairwise genetic distance (Γ_{st}) between locations

	Gimnesies	Azores	Madeira	Pitiüses	Lampedusa	NE Spain	W Italy
Azores	<i>0.001</i>						
Madeira	<i>0.003</i>	<i>0.001</i>					
Pitiüses	0.041 ^{a,b}	0.027	0.017				
Lampedusa	0.067 ^b	0.059 ^a	0.170 ^{a,b}	0.006			
NE Spain	0.168 ^{a,b}	0.167 ^{a,b}	0.151 ^{a,b}	0.055 ^{a,b}	0.032		
W Italy	0.256 ^{a,b}	0.215 ^{a,b}	0.170 ^{a,b}	0.108 ^{a,b}	0.056	<i>0.004</i>	
E Italy	0.340 ^{a,b}	0.287 ^{a,b}	0.231 ^{a,b}	0.173 ^{a,b}	0.101 ^{a,b}	<i>0.022</i>	<i>0.010</i>

The cluster of Azores, Madeira and Gimnesies is well differentiated from the cluster of NE Spain, W Italy and E Italy. Italic types show comparisons within clusters. Bold types highlight statistically significant differences between members of different clusters

^aChi-square test ($P < 0.05$)

^b S_{mn} test ($P < 0.05$)

when data were weighted, thus producing a more likely scenario.

A second source of uncertainty is the possible existence of temporal variations in the haplotype frequencies at the considered foraging grounds that may confuse with differences in the relative contribution of the considered rookeries. This had not been a source of concern if all the foraging grounds had been sampled simultaneously; However, this was not the case, as samples were collected from 1999 to 2003 but at different time spans at each foraging ground. Bass et al. (2004) found no annual differences in the genetic composition of a foraging ground at the Pamlico-Albermale Estuarine Complex in North Carolina during a 3-year sampling period, thus suggesting that temporal differences in the genetic composition of foraging grounds might occur only at a much higher temporal scale. However, one or two nesting season failures at a particular rookery due to stochastic phenomena (e.g. as a result of hurricanes) may cause significant changes to the production of nesting areas that might affect the composition of the foraging grounds. There is no way to be sure that the data here used are not biased by non-simultaneous collection of samples, although the range of turtle length from every foraging ground is large enough to guarantee that several cohorts are being sampled and hence that the effects of recruitment failure in a few years will be smoothed by successful recruitment in other periods. Nevertheless, haplotype frequencies at each foraging grounds and the relative contribution of each rookery to every foraging ground should not be considered as fixed values, but are expected to fluctuate due to stochastic changes in the haplotype frequencies at nesting sites and also due to annual variations in the reproductive success at each rookery.

Regardless of those shortcomings, there is no doubt that mixing of Atlantic and Mediterranean turtles within the western Mediterranean is not homogeneous in the light of the statistically significant differences observed in the genetic structure of several western Mediterranean foraging grounds. Furthermore, the Gimnesies Islands clustered with the Azores and Madeira, and not with the Pitiüses Islands, the north-eastern Spain and the Italian foraging 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. Differences in habitat selection between oceanic and neritic stage turtles cannot be either invoked to explain the structuring within the western Mediterranean as foraging grounds in the western Mediterranean differing in haplotype frequencies do not differ in average turtle length. Conversely, foraging grounds not differing in haplotype frequencies differ in average length (eastern Italy when compared with western Italy and north-eastern Spain).

Thus, differences in haplotype composition within the western Mediterranean are proposed to 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. A simple diffusion pattern from these points fails to generate the above-reported structuring, as there is no significant correlation between genetic and geographic distances. Conversely, the pattern of surface sea currents and the distribution of water masses explain successfully the reported genetic heterogeneity.

High-salinity water from the Tyrrhenian Sea flows along the western coast of Italy until it reaches the Ligurian Sea (Millot 1987, 2001; Pinardi and Masetti 2000). There, the Liguro-Provençal current originates and flows westwards, 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, 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 would appear to be a shortcut that accelerates the transport of eastern Mediterranean turtles along the European shore to the western Mediterranean.

The Algerian current flows eastwards from the Strait of Gibraltar to the Sicilian channel (Fig. 1; Millot 1987,

2001; Pinar di 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 (López-Jurado 1990) 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 may be explained by the Liguro-Provenzal current having 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). A possible consequence is that 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 (Fig. 1), 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).

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). 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. The stock of turtles nesting in the eastern Mediterranean (Margaritoulis et al. 2003) is much more reduced than that in the Atlantic rookeries (Ehrhart et al. 2003). Given that immature turtles from the former region that enter the western Mediterranean concentrate in the foraging grounds off the European shore, a reduction in turtle by-catch and mortality in this area should be considered a priority to protect the eastern Mediterranean population.

A further point that deserves discussion is the possible occurrence of loggerhead sea turtles from Mediterranean rookeries off Madeira and the Azores, as suggested by the mixing stock analysis. Considering the permanent eastward flow of surface water at the Strait of Gibraltar (Millot 1987) and the limited swimming and diving competence of immature loggerhead sea turtles shorter than 40 cm SCL (Bolten 2003), passage from the Mediterranean to the Atlantic is thought to be possible only for larger specimens. Although the average length of loggerhead sea turtles off the Azores is about 30 cm, much larger animals have been recorded there (Bjørndal et al. 2000) and elsewhere in the eastern Atlantic (Hays and Marsh

1997). However, size does not prove that they come from the Mediterranean, as tagging has revealed an extremely limited passage of turtles from the western Mediterranean to the adjoining Atlantic (Margaritoulis et al. 2003). Furthermore, those large specimens found in the mid-Atlantic foraging grounds might be Atlantic specimens coming back to their natal beaches after the oceanic phase. Clearly, this topic deserves further research.

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References

- Aguilar R, Mas J, Pastor X (1995) Impact of the Spanish swordfish longline fisheries on the loggerhead sea turtle *Caretta caretta* population in the western Mediterranean. In: Richardson JI, Richardson TH (eds) Proceedings of the 12th annual workshop on sea turtle biology and conservation. NOAA Technical Memorandum NMFS-SEFSC-361, pp 1–6
- Bass AL, Epperly SP, Braun-McNeill J (2004) Multi-year analysis of stock composition of a loggerhead turtle (*Caretta caretta*) foraging habitat using maximum likelihood and Bayesian Methods. *Conserv Genet* 5:783–796
- Bentivegna F (2002) Intra-mediterranean migration of loggerhead sea turtles monitored by satellite telemetry. *Mar Biol* 141:795–800
- Bjørndal KA, Bolten AB, Martins HR (2000) Somatic growth model of juvenile loggerhead sea turtle *Caretta caretta*: duration of pelagic stage. *Mar Ecol Prog Ser* 202:265–272
- Bolten AB (2003) Active swimmers-passive drifters: the oceanic juvenile stage of loggerheads in the Atlantic system. In: Bolten AB, Witherington BE (eds) *Loggerhead sea turtles*. Smithsonian Institution Press, Washington DC, pp 63–78
- Bolten AB, Bjørndal KA, Martins HR, Dellinger T, Biscoito MJ, Encalada SE, Bowen BW (1998) Transatlantic developmental migrations of loggerhead sea turtles demonstrated by mtDNA sequence analysis. *Ecol Appl* 8:1–7
- Bowen BW, Karl SA (1997) Population genetics, phylogeography, and molecular evolution. In: Lutz PL, Musick JA (eds) *The biology of sea turtles*. CRC Press, Boca Raton, pp 29–50
- Bowen BW, Bass AL, Soares L, Toonen J (2005) Conservation implications of complex population structure: lessons from the loggerhead turtle (*Caretta caretta*). *Mol Ecol* 14:2389–2402
- Broderick AC, Glen F, Godely BJ, Hays GC (2002) Estimating the number of green and loggerhead turtles nesting annually in the Mediterranean. *Oryx* 36:227–235
- Cardona L, Revelles M, Carreras C, San Félix M, Gazo M., Aguilar A (2005) Western Mediterranean immature loggerhead turtles: habitat use in spring and summer assessed through satellite tracking and aerial surveys. *Mar Biol* 147:583–591
- Carreras C, Cardona L, Aguilar A (2004) Incidental catch of loggerhead turtles *Caretta caretta* off the Balearic Islands (western Mediterranean). *Biol Conserv* 117:321–329
- Casale P, Laurent L, Gerosa G, Argano R (2002) Molecular evidence of male-biased dispersal in loggerhead turtle juveniles. *J Exp Mar Biol Ecol* 267:139–145

- Crouse DT, Crowder LB, Caswell H (1987) A stage-based population model for loggerhead sea turtles and implications for conservation. *Ecology* 68:1412–1423
- Davenport J, Clough W (1986) Swimming and diving in young loggerhead sea turtles (*Caretta caretta* L.). *Copeia* 1986:53–57
- Ehrhart LM, Ogren H (2000) Estudios en hábitos de alimentación: captura y manejo de tortugas. In: Eckert KL, Bjorndal KA, Abreu-Grobois FA, Donnelly M (eds) Técnicas de investigación y manejo para la conservación de las tortugas marinas. Specialist Group on Sea Turtles IUCN/CSE Pub. No 4, pp 70–74
- Ehrhart LM, Bagley DA, Redfoot WE (2003) Loggerhead turtles in the Atlantic Ocean: geographic distribution, abundance and population status. In: Bolten AB, Witherington BE (eds) Loggerhead sea turtles. Smithsonian Books, Washington DC, pp 157–174
- Encalada SE, Bjorndal KA, Bolten AB, Zurita JC, Schroeder B, Possardt E, Sears CJ, Bowen BW (1998) Population structure of loggerhead turtle (*Caretta caretta*) nesting colonies in the Atlantic and Mediterranean as inferred from mitochondrial DNA control region sequences. *Mar Biol* 130:567–575
- Engstrom TN, Meylan PA, Meylan AB (2002) Origin of juvenile loggerhead turtles (*Caretta caretta*) in a tropical developmental habitat in Caribbean Panamá. *Anim Conserv* 5:125–133
- Estrada M, Vives F, Alcaraz M (1985) Life and productivity in the open sea. In: Margalef R (ed) The western Mediterranean. Pergamon Press, London, pp 148–197
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98
- Hays GC, Marsh R (1997) Estimating the age of juvenile loggerhead sea turtles in the North Atlantic. *Can J Zool* 75:40–46
- Hudson RR (2000) A new statistic for detecting genetic differentiation. *Genetics* 155:2011
- Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2: Molecular evolutionary genetics analysis software. *Bioinformatics* 17:1244–1245
- Laurent L, Lescure J, Excoffier L, Bowen B, Domingo M, Hadjichristophorous M, Kornaraki L, Trabuchet G (1993) Genetic studies of relationships between Mediterranean and Atlantic populations of loggerhead turtle *Caretta caretta* with a mitochondrial marker. *C r Acad Sci III-Vie* 316:1233–1239
- Laurent L, Casale P, Bradai MN, Godley BJ, Gerosa G, Broderick AC, Schroth W, Schierwater B, Levy AM, Freggi D, Abd El-Mawla EM, Hadoud DA, Gomati HE, Domingo M, Hadjichristophorous M, Kornaraki L, Demirayak F, Gautier CH (1998) Molecular resolution of marine turtle stock composition in fishery bycatch: a case study in the Mediterranean. *Mol Ecol* 7:1529–1542
- López-Jurado JL (1990) Masas de agua alrededor de las Islas Baleares. *Bol Inst Esp Ocean* 6:3–20
- Malanotte-Rizzoli P, Manca BB, d'Alcala MR, Theocharis A, Bergamasco A, Bregant D, Budillon G, Civitarese G, Georgopoulos D, Michelato A, Sansone E, Scarazzato P, Souvermezoglou E (1997) A synthesis of the Ionian Sea hydrography, circulation and water mass pathways during POEM Phase 1. *Prog Oceanogr* 39:153–204
- Margaritoulis D, Argano R, Baran I, Bentivegna F, Bradai MN, Camiñas JA, Casales P, De Metrio G, Demetropoulos A, Gerosa G, Godley BJ, Haddoud DA, Houghton J, Laurent L, Lazar B (2003) Loggerhead turtles in the Mediterranean sea: present knowledge and conservation perspectives. In: Bolten AB, Witherington BE (eds) Loggerhead sea turtles. Smithsonian Books, Washington DC, pp 175–198
- Millot C (1987) Circulation in the western Mediterranean Sea. *Ocean Acta* 10:143–149
- Millot C (2001) Mesoscales and seasonal variabilities of the circulation in the western Mediterranean. *Dynamic Atmos Ocean* 15:179–214
- NMFS-SEFSC (National Marine Fisheries Service–South East Fisheries Science Center) (2001) Stock Assessments of loggerhead and leatherback sea turtles and assessment of the impact of the pelagic longline fishery on the loggerhead and leatherback sea turtles of the western North Atlantic. NOAA Technical Memorandum NMFS-SEFSC, 455, 343pp
- Norman JA, Moritz C, Limpus CJ (1994) Mitochondrial DNA control region polymorphisms: genetic markers for ecological studies of marine turtles. *Mol Ecol* 3:363
- Okuyama T, Bolker BM (2004) Combining genetic and ecological data to estimate sea turtle origins. *Ecol Appl* 15:315–325
- Owens D, Ruiz GW (1980) New methods of obtaining blood and cerebrospinal fluid from marine turtles. *Herpetologica* 1:17–20
- Pella J., Masuda M (2001) Bayesian methods for analysis of stock mixtures from genetic characters. *Fish Bull* 99:151–167
- Pinardi N, Masetti E (2000) Variability of the large-scale general circulation of the Mediterranean sea from observations and modelling: a review. *Palaeogeogr Palaeoclimat Palaeoecol* 158:153–173
- Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J Heredity* 86:248–249
- Rolf DA, Bentzen P (1989) The statistical analysis of mitochondrial DNA polymorphisms: X2 and the problem of small samples. *Mol Biol Evol* 6:539–545
- Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19:2496–2497
- Zaykin DV, Pudovkin AI (1993) Two programs to estimate significance of X2 values using pseudo-probability tests. *J Heredity* 84:152