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A comprehensive mitochondrial DNA mixed-stock analysis clarifies the composition of loggerhead turtle aggregates in the Adriatic Sea

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Abstract

Migration is one of several marine vertebrate traits increasingly affected by human encroachment. The Adriatic Sea is an important foraging and wintering site for loggerhead turtles (*Caretta caretta*), and one of the Mediterranean regions where they are most heavily impacted, particularly by fisheries bycatch. Conservation measures concern foraging and wintering areas and nesting grounds, and must, therefore, be informed by the natal origin of individuals in these mixed aggregates. Genetic diversity was investigated among 488 loggerheads found stranded or incidentally captured in fishing gear across the Adriatic and the origin of individuals was assessed through mixed-stock analysis based on comparison of 755 bp of mitochondrial DNA control region sequence. In addition, we highlight the importance of the Gulf of Manfredonia (Apulia, Italy), and of the northeastern Adriatic—a previously genetically undescribed foraging ground. No significant genetic divergence was recorded among sampling areas, between turtles sampled in cold and warm months or between adults and juveniles. The distribution of turtles across the Adriatic Sea appeared not to depend on individual origin. Rookeries in western Greece and Crete provided the most important contributions to the Adriatic mixed stock. In particular, the Greek populations were the most abundant locally in the Gulf of Manfredonia, so they are likely to experience an even greater impact than previously thought because of the severe fishery bycatch levels in this area. This study also provides an example of how substantial increases in sample sizes permit a relatively comprehensive testing of genetic structure across groupings in foraging aggregations.

Introduction

Patterns of dispersal and migration in marine vertebrates are known to embrace long-distance movements to breeding and nesting areas, foraging, and wintering grounds. Unfortunately, connectivity between habitats that play important roles in the life cycle of an organism can be disturbed or disrupted by a plethora of anthropogenic threats (Halpern

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et al. 2007). Bycatch, the incidental capture of non-target species in fisheries, is a prevalent cause of mortality particularly in the Mediterranean Sea, where marine species like sea turtles migrate on a regular basis from foraging to nesting grounds of great importance for population survival (Casale and Margaritoulis 2010; Fortuna et al. 2010; Coll et al. 2012; Luschi and Casale 2014).

The Adriatic Sea is one of the main foraging areas in the Mediterranean basin for marine wildlife, and in particular the loggerhead sea turtle *Caretta caretta*. The shallow (< 100 m) waters of the northern Adriatic are highly productive (Agostini and Bakun 2002) and a main neritic foraging and wintering ground for *C. caretta* (Margaritoulis et al. 2003; Casale et al. 2004; Lazar et al. 2004; Casale et al. 2010; Lazar et al. 2011; Luschi and Casale 2014). The southern Adriatic is mostly oceanic, has a wide depression more than 1200 m deep (Cushman-Roisin et al. 2001), and is regarded as an important area for early life stage development, although all size classes are present throughout (Casale et al. 2010; Casale and Mariani 2014).

An additional, important neritic foraging area has recently been described in the Gulf of Manfredonia (Italy), off the Apulian coast, and directly west of the South Adriatic trench. This region is frequented by turtles of different size classes showing a certain degree of site fidelity (Casale et al. 2012; Casale and Simone 2017).

Describing the connectivity between natal areas and foraging grounds represents a major challenge in sea turtle research and conservation. A variety of different tools have been adopted to investigate the long-range migration of chelonians, including capture-mark-recapture, satellite tracking, and genetic analysis. Molecular tools, in particular, can provide a thorough understanding of the natal origin of foraging stocks and, among others, give insights into how anthropogenic threats affect wild populations occurring in specific marine areas and/or nesting grounds (Hamann et al. 2010; Rees et al. 2017). Furthermore, genetic structure was among the main biological parameters involved in the recent designation of regional management units (RMUs) as groups of nesting populations on possible, independent evolutionary trajectories (Wallace et al. 2010).

Flipper tagging and satellite tracking studies of loggerheads breeding in Greece have shown the importance of the Adriatic foraging grounds for the Greek nesting populations (Lazar et al. 2004; Zbinden et al. 2008), but comparable studies from other Mediterranean nesting sites are lacking. Similarly, population genetic studies conducted on *C. caretta* sampled in the Adriatic Sea have demonstrated a prevalence of individuals from Greece (Garofalo et al. 2013; Clusa et al. 2014). Molecular tracking of sea turtles is mainly based on interindividual variation at mitochondrial DNA (mtDNA) sequences, which occur at different frequencies in different rookeries—a result of population structuring due to natal homing (Bowen et al. 2005). Given this rookery baseline description, mixed-stock analysis (MSA) can then be used to estimate the relative contribution of different natal sites to foraging areas (e.g., Bass et al. 2004; Bowen et al. 2007; Bjorndal and Bolten 2008). However, loggerhead mtDNA population structure is rather weak in the Mediterranean, probably because of relatively recent colonization events from the Atlantic Ocean (Clusa et al. 2013). For this reason, MSA may have low power when using a relatively small sample size, which is less likely to include rare and more informative mtDNA haplotypes (Bolker et al. 2003). The previous attempts to characterize the genetic population structure of loggerhead turtles sampled in the Adriatic Sea were based on a relatively small sample size and did not include individuals from the northeastern Adriatic waters and the Gulf of Manfredonia as major loggerhead foraging sites (Casale et al. 2012).

A thorough assessment of the natal origin of loggerheads foraging in the Adriatic basin may have profound implications for our understanding of migratory patterns of turtles in the Mediterranean Sea. Different migration routes and displacement observed in tagged and satellite-tracked adult turtles may, in fact, depend on their rookeries of origin or on other factors such as ocean currents or different foraging strategies (Zbinden et al. 2011). Although juvenile movements and dispersal are mostly driven by marine currents, natal homing has been observed in juvenile sea turtles that show site fidelity to pelagic feeding areas near their rookery of origin or areas at similar latitudes (Avens et al. 2003; Bowen et al. 2004; Monzón-Argüello et al. 2009). If dispersal to foraging grounds depends on where individual turtles come from (i.e., location and/or size of rookery of origin), then genetic structuring is expected, for turtles from different rookeries would feed in different coastal areas. If, on the other hand, the migration of turtles from different rookeries to feeding sites is based on factors other than individual origin, then no genetic structure should be recorded across the Adriatic Sea. Moreover, there might be seasonal and/or body size differences when turtles of different origin congregate at specific foraging grounds.

In this study, we assessed the natal origin of the Adriatic foraging stock through MSA, using a comprehensive data set composed of loggerhead samples collected across the Adriatic Sea as a result of bycatch and stranding events off and along the coasts of Croatia, Slovenia, northern Italy, and the southern Italian region of the Gulf of Manfredonia. We conducted a population genetic analysis to characterize previously undescribed mtDNA haplotypes and investigate possible differences among turtles from the Adriatic foraging population. Using a relatively large sample size, we attempted an optimization of Bayesian MSA by (1) adopting a population abundance prior (Pella and Masuda 2001) to account for the relatively higher contribution of rookeries with a large number of nests, rather than relying on haplotype frequencies only; and (2) relaxing the assumption of equal possibility of reaching the foraging area using priors based on geographical distance between known Mediterranean rookeries and the Adriatic Sea.

Intensive human activity in the Adriatic Sea, including fisheries, shipping, and pollution, is considered a potential threat to sea turtle populations (Franzellitti et al. 2004; Casale 2011; Lazar and Gračan 2011). Comprehensive knowledge of the composition of the Adriatic foraging stock will help to inform decision-making processes aimed at identifying marine protected areas and mitigating the anthropogenic impact on sea turtle survival. An assessment of migratory routes from the main contributing rookeries can provide background data for revising regional policies on the use of fishing gear particularly harmful to loggerheads, such as set nests and bottom trawlers—responsible for most bycatch and likely one of the main factors affecting survival rates of *C. caretta* in the Adriatic Sea (Casale et al. 2004, 2012, 2015).

Materials and methods

Sample collection

A total of 488 *C. caretta* samples were collected between 1995 and 2015 in the Adriatic Sea off or along the coasts of Slovenia, Croatia, and northern and southern Italy (Fig. 1). Blood and skin biopsies were taken from live bycaught turtles, while muscle and skin were obtained from necropsies of stranded or floating individuals. Sea turtles sampled alive were tagged before release to avoid pseudoreplication. Our sample set included both juveniles and adults with the curved carapace length ranging from 8.5 to 86.0 cm (see Electronic Supplementary Material Fig. S1 for the body size distribution of turtles sampled in the Adriatic Sea). Blood samples were stored at − 20 °C, while tissue samples were preserved in 95% ethanol at 4 °C or ambient temperature.

Genetic analysis

High-molecular-weight DNA was isolated using either a standard phenol/chloroform extraction protocol (Sambrook and Russel 2001), a NucleoSpin Tissue kit (Macherey–Nagel), or a QIAamp DNA Mini Kit (Qiagen). A fragment of the mtDNA was amplified by polymerase chain reaction (PCR) using the LTEi9 light-strand primer and H950 heavy-strand primer designed by Abreu-Grobois et al. (2006). The H950 primer anneals to the control region. The LTEi9 primer anneals between the threonine tRNA and proline tRNA genes, and in combination with H950 allows PCR amplification of an 887 bp mtDNA fragment. PCR was conducted in a total volume of 15 µl with 100 ηg of total DNA, $1 \times$ PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, and 0.5 units of *Taq* DNA polymerase. Thermal profiles consisted of an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C, and 1 min and 30 s at 72 °C, with a final extension step of 10 min at 72 °C. Amplicons were cycle-sequenced using BigDye Terminator v3.1 chemistry (Applied Biosystems) according to the manufacturer's protocol, and cycle sequencing reactions were resolved on Applied Biosystems 3100 Avant and 3130xl genetic analyzers.

Data analysis

Raw sequence chromatographs from both strands were edited and aligned to the loggerhead turtle mtDNA sequence using GENEIOUS 8.0.4 (Biomatters Ltd). The consensus sequence consisted of 755 bp of the mtDNA control region after trimming of tRNA gene sequences. We also trimmed sequences down to 380 bp to match the short mtDNA control
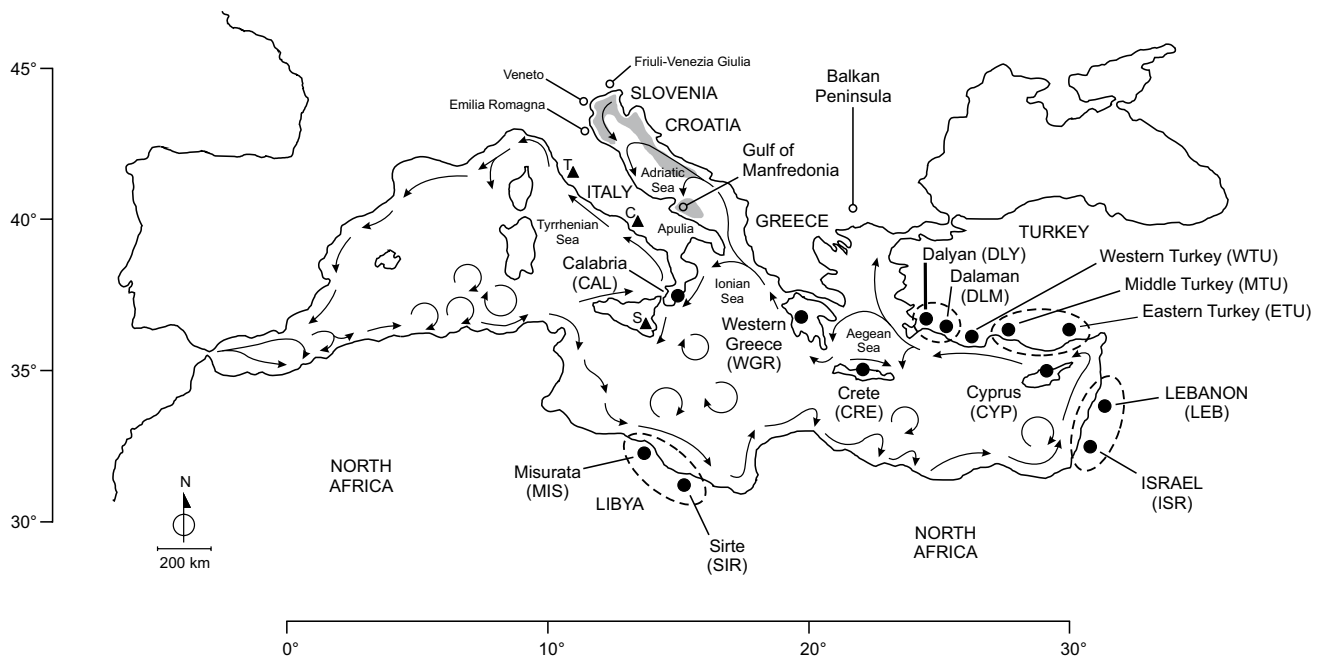
Fig. 1 Map and geographical coordinates of the study area. Samples were collected from live bycaught turtles, as well as stranded and floating individuals, either dead or alive, in the Adriatic Sea (grey areas). Black circles show the locations of Mediterranean rookeries considered in this study as potential natal sites. Black triangles mark the location of the occasional Italian nesting sites of Tuscany (T), Campania (C), and Sicily (S) described in our study. Dashed circles include the rookeries pooled for mixed-stock analysis. Arrows show the main sea surface currents

region fragment described by Norman et al. (1994) and compared sequence diversity parameters to values reported for *C. caretta* in the previous studies. Mitochondrial haplotypes were classified by comparison to the mtDNA sequences available on the Archie Carr Center for Sea Turtle Research database (ACCSTR; http://accstr.ufl.edu/files/cclongmtdna.pdf). Relationships among haplotypes were inferred using an unrooted haplotype network based on the TCS inference approach of statistical parsimony implemented in POPART (Leigh and Bryant 2015). The method links haplotypes with the smaller number of differences as defined by a 95% confidence criterion (Templeton et al. 1992; Clement et al. 2000).

Mitochondrial control region sequence diversity was inferred by the number of haplotypes (*k*), number of polymorphic sites (*p*), haplotype diversity (*h*), and nucleotide diversity (*π*) using ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010). The extent of mtDNA control region differentiation was investigated by principal coordinate analysis (PCoA) of a standardized pairwise haplotype distance matrix using GenAlEx 6.503 (Peakall and Smouse 2012). We then assessed the average number of pairwise differences in haplotype frequencies among turtles using the Φ-statistics of Excoffier et al. (1992) implemented in ARLEQUIN 3.5.2.2. The statistical significance of the Φ_{ST} values was obtained after 10,000 haplotype permutations. Following a preliminary pattern of haplotype clustering recovered by PCoA, Φ-statistics was performed by pooling samples according to sampling region, sampling season, and body size. The Adriatic Sea can be divided into a northern, central, and southern basin—each with distinct topographic, bathymetric, and dynamic features (Artegiani et al. 1997; Cushman-Roisin et al. 2001). Similarly, the eastern coast is generally high and rocky, whereas the western coast is low and mostly sandy. Such differences may be mirrored by latitudinal and longitudinal patterns of genetic divergence as recorded in several studies on marine vertebrates of the Adriatic (e.g., Garoia et al. 2004; Gaspari et al. 2015; Ruggeri et al. 2016). Geographical subdivision, therefore, considered a southern Adriatic region (southern Italy) and a northern group (Slovenia, Croatia, northern Italy), as well as a western Adriatic (Italy) and eastern Adriatic (Slovenia and Croatia) area. The seasonal partitioning divided samples collected during relatively colder months (November–May) from those obtained during warmer periods (June–October), the latter also being the nesting season for most Mediterranean populations. The differences between warmer and colder months in terms of loggerhead sea turtle distribution as well as the frequency of by-catch events were reported for the Adriatic Sea by Lazar et al. (2002). Moreover, sea temperature is known to affect the migratory strategies of *C. caretta* from nesting beaches to foraging grounds, as well as their seasonal habitat choice (e.g., Hawkes et al. 2007; Schofield et al. 2009). Cold months were defined as those

showing average water temperatures lower than the average annual temperature recorded in the Adriatic Sea across 34 locations in Italy, Slovenia, and Croatia from 2013 to 2015 (available at http://seatemperature.info/adriatic-sea-water-temperature.html). Warm months were those with average temperatures equal to or higher than the annual average. Finally, sequences were analyzed by dividing our sample set into two sea turtle body size classes defined according to the individual ability of swimming independently of water currents. Body size can affect the swimming ability of turtles and, therefore, their distribution under different sea-current speeds (Revelles et al. 2007). The mean speed of Adriatic currents, 0.30 m s^{-1} (Poulain 2001), was used to calculate the critical swimming speed (U_{crit}) which a turtle of a certain body size can sustain without enduring muscular fatigue as follows: $U_{crit} = 1.763$ SCL $- 0.262$ (Revelles et al. 2007; Monzón-Argüello et al. 2009), where SCL is the straight carapace length (Bolten 1999). Turtles with an SCL of 32 cm or larger were defined as individuals capable of swimming independently of sea currents, while turtles with an SCL of less than 32 cm were defined as juveniles (Casale et al. 2005) whose movements were more likely to have been affected by currents. Conversion from curved to straight carapace length was conducted following the method described by Casale et al. (2017).

An MSA was performed using the Bayesian approach implemented in the MIXSTOCK package in R (Bolker and Okuyama 2014). The package compares mtDNA sequences from one or more mixed populations with baseline haplotypes from source populations (rookeries). We used the foraging ground centric approach to estimate the posterior distribution with 95% confidence intervals of unknown proportions of loggerhead turtle rookeries in the Adriatic foraging stock (i.e., the proportion of Adriatic sea turtle stock originating from distinct rookeries) (Pella and Masuda 2001; Bolker et al. 2003).

We assessed the composition of the Adriatic stock using a baseline of 10 rookeries from the Atlantic Ocean and 13 rookeries from the Mediterranean Sea (Fig. 1; Electronic Supplementary Material Table S1). Baseline sequences from the island of Zakynthos and the bays of Kyparissia and Lakonikos in the Peloponnese Peninsula were pooled into a single western Greece group according to Carreras et al. (2014) and non-significant pairwise Φ_{ST} values (ranging from $- 0.01$ to $- 0.04$; $P > 0.05$). Only short mtDNA control region sequences of less than 500 bp were available from the small rookeries of Lampedusa (Italy) and the Kuriat islands (Tunisia) (Laurent et al. 1998; Chaieb et al. 2010), so these rookeries were not included in the baseline. Haplotypes from single Mediterranean nests were also not part of our MSA for they could not be ascribed to a geographically defined rookery with a specific haplotype frequency. A few Mediterranean sites

that receive sporadic nesting have the haplotype CC-A10.4 (Garofalo et al. 2016a; Maffucci et al. 2016), previously found with a 0.1% frequency in the Central East Florida rookery (Shamblin et al. 2012) and likely to occur in other Mediterranean rookeries. Samples carrying the CC-A10.4 haplotype were also excluded from the MSA in order not to overestimate the Atlantic contribution in a baseline where this haplotype is found in one Atlantic rookery only.

An MSA was also performed by pooling rookeries into Mediterranean, Northwest Atlantic, and Northeast Atlantic RMUs (Wallace et al. 2010; Naro-Maciel et al. 2014). Subsequent analyses were conducted by (1) removing the contribution of the Atlantic nests from the baseline and (2) pooling 8 out of the 13 Mediterranean rookeries into four clusters, which included the two sites in Libya, Dalaman, and Dalayan in Turkey, middle, and eastern Turkey and the sites in Lebanon and Israel, respectively (Fig. 1; Electronic Supplementary Material Table S2). A similar subdivision of rookeries was proposed by Shamblin et al. (2014), whereby rookeries in Lebanon, Israel, Cyprus, eastern, and Middle Turkey were pooled into a single reference group. A Φ_{ST} test of pairwise differences in haplotype frequencies performed using ARLEQUIN 3.5.2.2 recorded no significant differences between rookeries pooled for MSA (Electronic Supplementary Material Table S3).

Priors were based on either of the following: (1) equal contribution from each rookery; (2) contribution weighted by population abundance; (3) contribution weighted by shortest swimming distance between rookeries and foraging ground; or (4) contribution weighted by both abundance and distance. The average number of nests per year was used as an estimate of population abundance (Electronic Supplementary Material Table S1). In the first MSA, where all 23 individual rookeries were considered, we used an equal contribution prior. On the other hand, all four different priors were used after pooling the eight rookeries from Libya, Lebanon, Israel, and Turkey into four distinct clusters. We also performed an MSA using the 380 bp mtDNA control region sequences to compare data obtained using mtDNA fragments of different length. As for the MSA based on the longer, 775 bp sequence fragment, the contribution of the Atlantic rookeries was close to zero (Electronic Supplementary Material Fig. S3). Subsequent analyses were, therefore, conducted following the same procedure used for the longer mtDNA control region sequence. We used a number of Markov chains equal to the number of source populations with 200,000 iterations and a 50% burn-in. Convergence of Markov chains was assessed according to the Gelman–Rubin criterion whereby a value lower than 1.2 for all parameters is taken as a general rule of thumb to test for between-chain variance being relatively smaller than within-chain variance, and for chains to converge (Gelman and Rubin 1992).

Results

Mitochondrial DNA control region sequence diversity

A total of 14 haplotypes were characterized in the *C. caretta* Adriatic foraging stock (Table 1). Four haplotypes (CC-A2.1, CC-A3.1, CC-A10.4, and CC-A20.1) were found in both Atlantic and Mediterranean rookeries and accounted for 91.6% of the Adriatic turtles. Among these, CC-A2.1 was the most abundant but the least informative as it is almost ubiquitous in Mediterranean and Atlantic rookeries. Haplotype CC-A10.4 is found in occasional Tyrrhenian nesting sites in the Mediterranean Sea (Garofalo et al. 2016a; Maffucci et al. 2016; Fig. 1). Two out of three individuals carrying haplotype CC-A10.4 were sampled in the southern Adriatic, off the Apulian coast. Eight exclusively Mediterranean haplotypes (CC-A2.8, CC-A2.9, CC-A6.1, CC-A26.1, CC-A29.1, CC-A31.1, CC-A32.1, and CC-A53.1) accounted for 8.2% of the total Adriatic sequences. Eleven (2.3%) loggerheads carried haplotype CC-A2.8, which is specific to the Cretan rookery, while 10 (2.0%) individuals had haplotypes CC-A6.1 and CC-A32.1 endemic to western Greece. Two individuals sampled in Slovenia carried haplotype CC-A29.1, found only in the Israeli rookery. Haplotype CC-A26.1, exclusive to Sirte (Libya), was recorded in three individuals from the northern Adriatic and one individual from the Apulian waters. We found only one turtle with Turkish haplotype CC-A53.1, recovered in Slovenian waters. Similarly, only one haplotype CC-A31.1, assigned to Calabrian and Greek rookeries and recently found in occasional nesting sites in Sicily (Garofalo et al. 2016b), was found in the northwestern Adriatic. Only 0.2% of the foraging stock (one juvenile turtle sampled along the Apulian coast) carried the Atlantic haplotype CC-A1.1.

One new haplotype was recorded in one individual sampled off the coast of Slovenia. The new haplotype differed from CC-A2.1 for one A-to-G transition at nucleotide site 15,648 of the published *C. caretta* mitochondrial genome sequence (Drosopoulou et al. 2012) (Fig. 2). The new sequence was named CC-A73.1 after submission to ACCSTR and deposited in GenBank under accession number MF182628. Haplotype CC-A73.1 was not considered in the MSA as its rookeries of origin have yet to be identified.

The number of haplotypes, number of polymorphic sites, and haplotype and nucleotide diversities were significantly higher in turtles sampled in the northern versus the southern Adriatic. Similarly, genetic diversity indices were higher in the eastern than in the western Adriatic (Table 2; Electronic Supplementary Material Table S4).

Table 1 Number (*n*) and frequency (%) of haplotypes of *C. caretta* sampled in the Adriatic Sea, and rookeries of origin of haplotypes (Clusa et al. 2014)

| Haplotype	*n*	%		Rookery
CC-A1.1	1	0.2	Apulia (1)	Mediterranean: – Atlantic: NOR, CEF, SEF, DRT, SWF, CWF, NWF
CC-A2.1	410	84.0	Friuli-Venezia Giulia (7), Veneto (66), Emilia Romagna (110), Apulia (137), SLOVENIA (48), CROATIA (42)	Mediterranean: MIS, SIR, ISR, LEB, CYP, ETU, MTU, DLM, DLY, CRE, WGR, CAL Atlantic: CEF, SEF, SAL, DRT, QMX, SWF, CWF, NWF, CPV
CC-A2.8	11	2.3	Friuli-Venezia Giulia (1), Veneto (2), Emilia Romagna (3), Apulia (3), SLOVENIA (2)	Mediterranean: CRE Atlantic: –
CC-A2.9	11	2.3	Veneto (3), Emilia Romagna (1), Apulia (2), SLOVENIA (5)	Mediterranean: MIS, SIR, ISR Atlantic: –
CC-A3.1	32	6.6	Veneto (1), Emilia Romagna (12), Apulia (11), SLOVENIA (5), CROATIA (3)	Mediterranean: MIS, SIR, LEB, ETU, WTU, DLM, DLY Atlantic: CEF, SEF, SAL, QMX, SWF, CWF, NWF
CC-A6.1	5	1.0	Emilia Romagna (3), Apulia (1), CROATIA (1)	Mediterranean: WGR Atlantic: –
CC-A10.4	3	0.6	Veneto (1), Apulia (2)	Mediterranean: Tuscany[a], Campania[a] Atlantic: CEF
CC-A20.1	1	0.2	Friuli-Venezia Giulia (1)	Mediterranean: CAL Atlantic: CEF, SEF, SWF, CWF
CC-A26.1	4	0.8	Veneto (1), Emilia Romagna (1), Apulia (1), CROATIA (1)	Mediterranean: SIR Atlantic: –
CC-A29.1	2	0.4	SLOVENIA (2)	Mediterranean: ISR Atlantic: –
CC-A31.1	1	0.2	Veneto (1)	Mediterranean: CAL, WGR, Sicily[a] Atlantic: –
CC-A32.1	5	1.0	Emilia Romagna (1), Apulia (1), SLOVENIA (2), CROATIA (1)	Mediterranean: WGR Atlantic: –
CC-A53.1	1	0.2	SLOVENIA (1)	Mediterranean: ETU, MTU Atlantic: –
CC-A73.1	1	0.2	SLOVENIA (1)	This study

Mediterranean rookeries: Acronyms as in Fig. 1. Atlantic rookeries: South Carolina and Georgia (NOR), Central-eastern Florida (CEF), South-eastern Florida (SEF), Cay Sal Bank, Bahamas (SAL), Dry Tortugas, Florida (DRT), Isla Cozumel and Mainland Quintana Roo, Mexico (QMX), South-western Florida (SWF), Central-western Florida (CWF), North-western Florida (NWF), Cape Verde (CPV)

[a]Occasional nesting sites recorded in Italy (Garofalo et al. 2016a, b; Maffucci et al. 2016)

Eight polymorphic sites were recorded in turtles from the Apulian waters after removal of a single individual carrying haplotype CC-A1.1, which differed by 41 mutations from CC-A2.1 (see Electronic Supplementary Material Table S5 for genetic diversity measures obtained with the full data set). Haplotype and nucleotide diversities calculated for the short (380 bp) control region fragment were 0.22 ± 0.00 and 0.0006, respectively, for the northern Adriatic and 0.20 ± 0.00 and 0.0013, respectively, for the southern Adriatic. Molecular diversity indices were also significantly higher in turtles sampled during warmer months than in the colder season. A lower number of haplotypes and polymorphic sites were observed in adults versus juveniles, which accounted for 15% of our sample set. These values were recorded after removing the one individual carrying haplotype CC-A1.1 from the analysis.

The first and second coordinates of the PCoA explained 67.5 and 11.8% of the total variance, respectively. A pattern of haplotype differentiation similar to the one described by the statistical parsimony network was recovered, whereby CC-A26.1 and CC-A32.1 were the most divergent from the other haplotypes. However, the PCoA failed to recover a clear clustering between haplotypes exclusive of either the eastern (CC-A20.1, CC-A29.1, CC-A53.1, and CC-A73.1) or western (CC-A10.4, CC-A31.1, and CC-A32.1) Adriatic, and between haplotypes recorded in adults (CC-A31.1) or juveniles only (all others but CC-A2.1 and CC-A3.1). There were no haplotypes found only in warm or cold months or only in the northern or southern Adriatic (Electronic Supplementary Material Fig. S4). There was no significant differentiation in the average number of pairwise differences in haplotype frequencies as computed using Φ-statistics between the North and South Adriatic groups, West and East Adriatic groups, winter and summer groups, or body size clusters (Table 3). MSA was, therefore, conducted by pooling all samples in a single Adriatic stock.
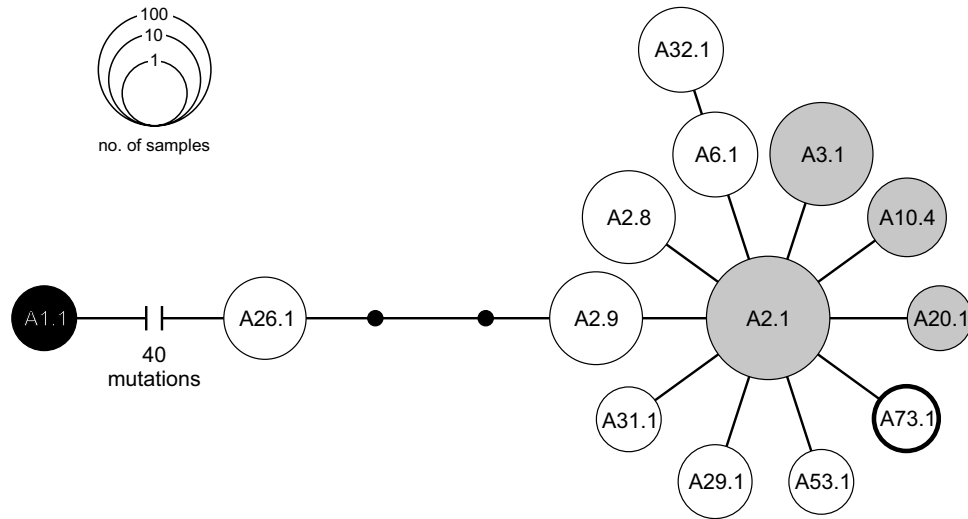
Fig. 2 Unrooted haplotype network of *C. caretta* estimated by statistical parsimony analysis. The areas of the circles are proportional, on a logarithmic scale, to the number of samples carrying a specific haplotype (see Table 1). White and black circles refer to haplotypes recorded in Mediterranean and Atlantic rookeries, respectively. Grey circles are haplotypes found in both Mediterranean and Atlantic rookeries. The white, thick circle is the haplotype recorded in this study and not yet assigned to a rookery. Small solid circles are intermediate haplotype states that were not observed in our data set

Table 2 Genetic diversity measures of *C. caretta* obtained after pooling samples by sampling region, season, and body size (straight carapace length—SCL)

	n	k	p	$h \pm$ SE	π
Sampling region					
North Adriatic	328	12	12	0.30 ± 0.01	0.0005
South Adriatic	158	8	8	0.24 ± 0.02	0.0004
West Adriatic	364	9	9	0.26 ± 0.00	0.0004
East Adriatic	123	11	11	0.37 ± 0.00	0.0006
Season					
Cold	153	6	7	0.22 ± 0.00	0.0004
Warm	271	13	13	0.32 ± 0.00	0.0005
Body size					
> 32 cm SCL	377	11	11	0.24 ± 0.00	0.0004
< 32 cm SCL	68	8	8	0.41 ± 0.01	0.0009

n number of turtles sampled, k number of haplotypes, p number of polymorphic sites, h haplotype diversity, π nucleotide diversity
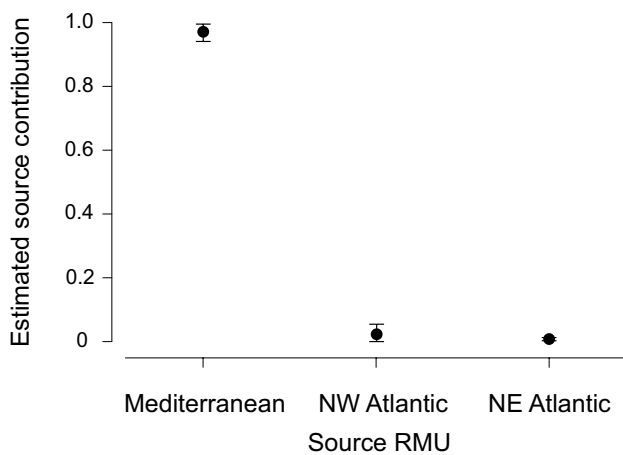
Adriatic loggerhead turtle stock composition

The mixed-stock analysis performed using a baseline of 23 rookeries and equal contribution prior revealed a contribution of the Atlantic rookeries close to zero. However, the analysis had a shrink factor higher than 1.2 and reported relatively large confidence intervals for Mediterranean rookeries (Electronic Supplementary Material Table S6, Fig. S2). In contrast, the MSA of the relative contribution of three distinct RMUs to the Adriatic stock exhibited a shrink factor < 1.2 and very narrow confidence intervals. The analysis showed a remarkable discrepancy between contribution estimates of Atlantic rookeries, all very close to zero, and contribution proportions of Mediterranean rookeries (Fig. 3).

The MSA performed without Atlantic rookeries satisfied the Gelman–Rubin criteria, with shrink factors < 1.2, and showed good chain convergence, albeit with wide confidence intervals recorded. According to the MSA with priors weighted by population abundance and distance from the rookeries, the western Greece rookery had the highest contribution to the Adriatic mixed stock ($P = 0.38$, 95% CI 0.14–0.67), followed by Crete ($P = 0.18$, 95% CI 1.65E-06

Table 3 Pairwise comparison matrix of φ_{ST} values among loggerhead sea turtles pooled by sampling region, season, and body size (straight carapace length—SCL)
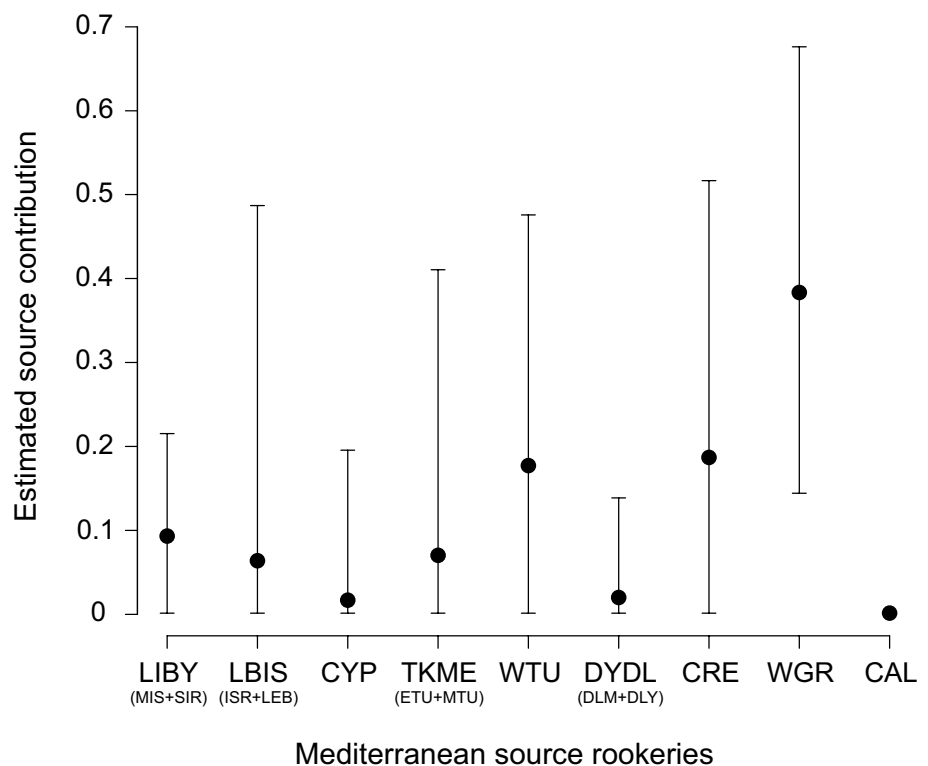
	North Adriatic		West Adriatic		Cold		> 32 cm
South Adriatic	− 0.002	**East Adriatic**	0.005	**Warm**	0.004	**< 32 cm**	0.012
	$P = 0.62$		$P = 0.11$		$P = 0.11$		$P = 0.09$

Fig. 3 Contribution proportions and 95% confidence intervals of the Mediterranean, Northwest Atlantic, and Northeast Atlantic regional management units to the Adriatic loggerhead turtle foraging stock estimated using an equivalent-contribution prior

to 0.51) and western Turkey ($P = 0.18$, 95% CI 3.81E-20 to 0.47). Much lower contributions were recorded for rookeries from the remaining Turkish sites, Libya, Lebanon, and Israel (Fig. 4; Electronic Supplementary Material Table S7). A similar pattern was observed when using an equal prior, as well as a prior based on contribution weighted by either population abundance or distance only (Electronic Supplementary Material Figs. S5–S7). Western Greece was

the most represented, while there was a slight increase in the contribution to the Adriatic stock from the rookeries of Israel and Lebanon when using an equal prior (Electronic Supplementary Material Table S8).

Analysis of the shorter, 380 bp mtDNA control region fragment recovered a similar pattern of rookery contribution to the Adriatic stock except for Crete, which had a contribution approximately 10% lower than the one recorded using the longer control region sequence (Electronic Supplementary Material Figs. S8–S11). This is because the short sequence does not include a polymorphism of haplotype CC-A2.8, which is distinctive of Cretan rookeries.

Discussion

The Adriatic Sea is one of the main Mediterranean European fishing grounds for both pelagic and demersal fisheries, in addition to being a very important feeding ground for *C. caretta*. This overlap highlights the urgent need for management actions to balance the exploitation of marine resources with the protection of vulnerable loggerhead turtles and their critical habitats across the Adriatic Sea. Describing the origin of loggerhead turtles frequenting the Adriatic and their genetic characterization can be particularly useful in the design of effective fishery management plans.

This study investigated the population genetic diversity of loggerhead turtles across the Adriatic Sea, using a

Fig. 4 Contribution proportions and 95% confidence intervals of nine rookeries and groups of rookeries from the Mediterranean Sea to the Adriatic loggerhead turtle foraging stock estimated using priors based on contribution weighted by both population abundance and distance between rookeries and foraging grounds. Acronyms of rookeries as in Fig. 1

comprehensive sample set from Slovenia, Croatia, and Italy. Stranded and bycaught individuals in Italian waters were obtained from the northern regions and from the southern area of the Gulf of Manfredonia—a particularly important foraging ground of conservation concern. Haplotype and nucleotide diversities were within the range of values recorded in the previous studies, despite such studies having been based on very small numbers of loggerheads sampled in the Adriatic Sea. For instance, Clusa et al. (2014) reported lower haplotype and nucleotide diversity values for juvenile *C. caretta* sampled in both the northern and southern Adriatic basins. Garofalo et al. (2013) recorded higher diversity values in the North Adriatic foraging ground, although the comparison was based on the short (380 bp) fragment of the mtDNA control region. The genetic diversity recovered in our study was, nevertheless, lower than the diversity values described for loggerheads sampled in other western and eastern Mediterranean foraging sites, probably because the Adriatic is geographically a semi-enclosed basin (Garofalo et al. 2013; Clusa et al. 2014).

Differences in genetic diversity measures were observed between the North and South Adriatic, West and East Adriatic, as well as between turtles of different body sizes or sampled in distinct seasons. While such differences may have been due to a discrepancy in sample size for the majority of comparisons, the eastern Adriatic had higher measures than the western Adriatic, despite the lower number of turtles sampled. Genetic divergence based on pairwise differences in haplotype frequencies was nil or negligible among sampling regions, between turtles sampled in winter and summer, and between adults and juveniles. The absence of a clear genetic structure across the Adriatic basin strongly suggests the absence of a genetic distinctiveness of turtles foraging across an area extending from the northernmost part of the Adriatic Sea, genetically described here for the first time, to the southern Gulf of Manfredonia. The observed lack of genetic differentiation supports the hypothesis that loggerhead turtles do not disperse across the Adriatic foraging ground based specifically on their natal origin.

MSA based on RMUs recorded a negligible contribution from Atlantic rookeries to the Adriatic stock, corroborating the previous studies on loggerhead turtle distribution in the Mediterranean basin (Garofalo et al. 2013; Clusa et al. 2014). On the other hand, nesting sites in western Greece and Crete were the most important rookeries contributing to the Adriatic foraging population. Geographical proximity to the Adriatic basin, sea currents, and size of the Greek populations increase the likelihood of presence of adults, juveniles, and hatchlings from Greek rookeries in the Adriatic Sea (Casale et al. 2007; Casale and Mariani 2014). Moreover, eastern Mediterranean currents flow counterclockwise from North Africa to Turkey and the Balkan Peninsula (Fig. 1). Waters from the Aegean and Ionian Sea reach the

northern Adriatic along the Croatian coastline and then flow back southwards to the Ionian Sea along the Italian peninsula (Millot and Taupier-Letage 2005; Cushman-Roisin et al. 2001). A likely migratory route to the Adriatic is, therefore, provided by sea currents flowing northwards from the Greek nesting grounds (Hays et al. 2010). Capture–mark–recapture and telemetry data from adult loggerhead turtles breeding in Mediterranean rookeries, and particularly in Greece, support genetic evidence of the extensive use of the Adriatic as a foraging ground for individuals from Greek nesting areas (Lazar et al. 2004; Luschi and Casale 2014). The fact that *C. caretta* from both the northern and the southern Adriatic foraging areas mainly originate from Greece is of particular conservation concern for the Greek population, which is likely to be affected by unmanaged Adriatic anthropogenic threats in greater proportion than previously estimated (Casale et al. 2004, 2010; Fortuna et al. 2010). In addition, increased numbers of nesting individuals or hatchlings from Greece would come under threat while feeding in the Adriatic, nullifying the conservation gains from the protection of nesting beaches.

Western Turkey had the third highest posterior probability of contributing to the Adriatic stock, along with the Libyan rookeries. The Libyan coastline is largely unsurveyed for the presence of nesting sites (Hamza 2010), meaning that the actual contribution to foraging areas might, in fact, be underestimated if a limited number of nests per year is used as the population abundance prior in the MSA. The Levantine coast (LBIS) nesting site had a surprisingly medium–high contribution, mainly as a result of two turtles recovered from the coast of Slovenia, in the northeastern Adriatic, carrying haplotype CC-A29.1, only recorded in Israeli rookeries to date. This haplotype might also occur in other, larger rookeries that have not yet been sampled or are yet undiscovered, in which case the LBIS contribution to the Adriatic stock would be relatively lower. In fact, although rookeries from Cyprus and the southern and western Turkish coasts (pooled in the TKME and DYDL clusters, respectively) are closer to the Adriatic Sea than the Israeli nesting sites and noticeably more abundant (see Electronic Supplementary Material Table S1), they provided a similar or smaller contribution than the Levantine rookeries. The possibility that haplotype CC-A29.1 could originate from rookeries other than the Israeli ones implies that unknown or poorly sampled nesting areas might be important to loggerhead recruitment yet to be quantified.

Contribution from Calabrian nesting sites was close to zero despite the relatively short geographical distance from the Adriatic Sea. This is most probably due to the small rookery size of Calabria and the sea currents flowing southwards along the Ionian coast of Italy, hindering migration to the Adriatic basin (Millot and Taupier-Letage 2005). Moreover, the rookery from Calabria shares haplotype CC-A31.1

with both western Greece and an occasional nesting site recently described in Sicily (Garofalo et al. 2016b). This makes detection of a Calabrian contribution to the Adriatic stock even more difficult. The same is true for another rare haplotype, CC-A10.4, described only in occasional nesting sites along Tyrrhenian beaches (Garofalo et al. 2016a; Maffucci et al. 2016). The three individuals carrying CC-A10.4 are likely to originate from Mediterranean colonies where CC-A10.4 has yet to be described rather than from Tyrrhenian rookeries. The CC-A10.4 haplotype may be part of the CC-A10 haplogroup (based on sequencing of 380 bp of the mtDNA control region) recorded in Greece by Laurent et al. (1998) but never observed afterwards in the same rookery that was also the major source of our Adriatic mixed stock.

In this study, we tried to optimize the MSA resolution primarily using a relatively large sample size. Although approximately 90% of the Adriatic stock included the most common CC-A2.1 and CC-A3.1 haplotypes, we found additional rare, rookery-specific sequences with respect to the previous studies based on much smaller sample sizes. We recorded haplotypes CC-A26.1 and CC-A29.1 so far described in the Libyan rookery of Sirte and the Israeli nesting sites, respectively. Haplotype CC-A20.1, which occurs in several Atlantic rookeries and only in one Mediterranean nesting site (Calabria, southern Italy), was recorded in one turtle from the northeastern Adriatic. Moreover, one turtle sampled in the northern Adriatic, off the coast of Slovenia had haplotype CC-A53.1, which is specific to eastern and central Turkish rookeries and never previously reported in the Adriatic (Yilmaz et al. 2012; Garofalo et al. 2013; Clusa et al. 2014).

We further improved the MSA performance in detecting mixed stocks from the Mediterranean baseline by removing and pooling baseline rookeries. We found that using nine single and clustered rookeries (namely the Libyan, Levantine, Cypriot, eastern, western and westernmost Turkish, Cretan, western Greek, and Calabrian) resulted in a relatively more informed baseline than using a set of reference sequences from 13 separate nesting grounds. Shamblin et al. (2014) pooled rookeries from Israel, Lebanon, Cyprus, and eastern and central Turkey into one single cluster despite significant Φ_{ST} values differentiating the Levantine and Cypriot nesting sites. Our scheme was similar to the one presented by Shamblin et al. (2014) except for retaining Cyprus, the two eastern Turkish sites (MTU and ETU) and the Levantine sites (LEB and ISR) as three distinct groups. The optimal grouping and/or splitting of rookery sites for baselines in MSA can, indeed, vary with geographical scale as well as conservation priorities. For instance, there may be compelling reasons to treat rookeries as demographically distinct for management purposes even in the absence of evidence of genetic differentiation. Formia et al. (2006) recommended recognition of Bioko

and Ascension Island as demographically distinct rookeries despite lack of genetic divergence, given that they are distant by approximately 3000 km. A lack of marker resolution can make decisions on baseline structure even more complex, particularly at finer scale, such as in the eastern Mediterranean basin. Our pooling scheme considered geographically and ecologically close rookeries with similar genetic profiles, as recovered by Φ-statistics. This resulted in a relatively finer definition of some of the natal ground contributions to the Adriatic stock. For instance, the first MSA conducted with a baseline of 13 Mediterranean rookeries resulted in relatively wide confidence intervals, particularly for the contribution of the nesting sites in Misurata. Pooling of the Libyan rookeries drastically reduced the confidence intervals of the posterior probability of contribution to the Adriatic stock. Finally, priors weighted by population abundance (average number of nests per year) and length of migratory routes resulted in more realistic contributions from small, geographically distant rookeries. Approximately 20 and 10% of the Adriatic stock was found to include turtles from Lebanon and Israel, respectively, when using equal priors and priors weighted for population abundance. These values were reduced to less than 10% when using priors weighted for both abundance and geographical distance between nesting and foraging grounds.

MSA of Mediterranean foraging grounds is, nonetheless, hampered by two fundamental issues. First, the Mediterranean baseline is not a comprehensive one. For instance, frequencies of certain haplotypes were either large with respect to rookery size or inconsistent with the distance between sampling area and nesting sites. This suggests that haplotypes such as CC-A29.1 and CC-A10.4 might also originate at rookeries other than the ones described to date. Moreover, our finding of a novel mtDNA control region haplotype implies that more nesting areas need to be sampled (or sample sizes increased) to attain an inclusive baseline for sample assignment, particularly along the largely unexplored coasts of Libya. Second, the resolution power of currently available mtDNA control region haplotypes seems inadequate to accurately describe unique rookeries and their fine-scale contributions to foraging grounds. Analysis of both mtDNA sequences and microsatellite allele frequencies can better recover patterns of genetic structure among nesting grounds (Carreras et al. 2007). For instance, an integrated approach proved to be a powerful tool for MSA and individual assignment of loggerhead turtle from western Mediterranean feeding grounds to either Atlantic or Mediterranean RMUs, whereby a 35% increase in robust assignment was recovered when using both markers rather than mtDNA alone (Carreras et al. 2011). Studies on interindividual variation using additional mtDNA and nuclear markers should, therefore, be explored, to better inform our understanding of rookery

dispersal and distribution throughout the Mediterranean (Duchene et al. 2012; Novelletto et al. 2016).

Notwithstanding the need for improved genetic sampling at several Mediterranean rookeries, our results confirm a high degree of connectivity between the Adriatic and western Greece and Crete, and, therefore, the importance of collaborative management efforts throughout the range of these populations. While migratory routes to and from the Adriatic deserve concerted conservation attention, genetic results indicate that foraging loggerheads do not exhibit preferential habitat use within Adriatic sub-regions. However, further research is needed to assess whether they may be able to shift foraging zones in response to local threats or whether depletion in more threatened areas such as the northern Adriatic and the Gulf of Manfredonia may be compensated by shifts in distributions of the foraging population.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care of animals were followed. This work was conducted under all necessary permits. Live bycaught sea turtles were released after sample collection according to the IUCN/SSC Marine Turtle Specialist Group guidelines (Eckert et al. 1999). None of the procedures used in the study met the criteria which would define them as "experiments" according to article 2 of the Council of European Communities Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. Approval by the institutional ethics committee (Commissione per l'Etica della Ricerca di Ateneo, University of Florence) was not required.

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