Journal of Experimental Marine Biology and Ecology 439 (2013) 15–24

Contents lists available at SciVerse ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Mitochondrial DNA reveals Pleistocenic colonisation of the Mediterranean by loggerhead turtles (*Caretta caretta*)

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ARTICLE INFO

Article history:
Received 23 December 2011
Received in revised form 15 October 2012
Accepted 17 October 2012
Available online 11 November 2012

Keywords:
Caretta caretta
Genetic structuring
Glacial refugia
Molecular clock
mtDNA
Phylogeography

ABSTRACT

As the loggerhead turtle (*Caretta caretta*) is a philopatric species with a strong genetic structure, the analysis of mtDNA can be used to track evolutionary and colonisation events. In this study we use a genetic approach to understand the population structure of *C. caretta* in the Mediterranean Sea and to test whether loggerheads could have colonised the Mediterranean during the Pleistocene and survived the cold phases in warm refugia. We amplified a long mtDNA D-loop fragment (815 bp) from 168 dead hatchlings sampled from a selection of rookeries in the Eastern Mediterranean: Libya, Israel, Lebanon, Cyprus and Greece. Previously published data from Turkey and Calabria (Southern Italy) were also included in the analyses. The population nesting in Libya emerged as the oldest population in the Mediterranean, dating from the Pleistocene ca. 65,000 years ago (20,000–200,000). This reveals that the Libyan population might have settled in the Mediterranean basin before the end of the last glacial period. The remaining nesting sites, except Calabria, were subsequently colonised as the population expanded. The populations nesting in Eastern Turkey and Western Greece settled ca. 30,000 years ago (10,000–100,000), whereas the remaining populations originated as a result of a more recent Holocenic expansion. As Calabria presented a unique Atlantic haplotype, found nowhere else in the Mediterranean, we consider this nesting site as the result of an independent colonisation event from the Atlantic and not the recent spread of Mediterranean populations. This reveals that the current genetic structure of *C. caretta* rookeries in the Mediterranean would be the result of at least two colonisation events from the Atlantic, the oldest one in Libya and a most recent in Calabria, combined with local extinctions during Pleistocenic glaciations and re-colonisations from glacial refugia in Libya, Eastern Turkey and Western Greece.

1. Introduction

The Pleistocene extended from 2.5 mya to 12 kya and was characterised by multiple glacial–interglacial cycles that caused dramatic changes in the distribution of organisms (Taberlet et al., 1998; Wilson and Eigenmann Veraguth, 2010). As ice sheets spread during glacial cycles, species often retreated towards the Equator although some populations survived in areas that acted as refugia (Haffer, 1982). Furthermore, a dryer climate and lower sea levels during glacial periods caused dramatic changes in species distribution even in areas that were not covered by ice (Hewitt, 1996; Maggs et al., 2008). When ice retreated due to post-glacial temperature rises, species re-expanded their distribution polewards, occupying previously inhospitable areas (Hewitt, 2000). These patterns are well established for terrestrial organisms, but the response to Pleistocenic glacial–interglacial cycles is still unclear for many marine species.

After the Messinian Salinity Crisis (5.33–5.59 mya), the Mediterranean basin was colonised by subtropical biota of Atlantic origin

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http://dx.doi.org/10.1016/j.jembe.2012.10.011

(Pérès, 1985). During the following climatic fluctuations, species distributions were affected by changes in the sea level, water temperature and salinity (Grant and Bowen, 1998). According to the fossil records, the most thermophilic groups became extinct during the first cold period of the Pleistocene and waves of extinction and invasion changed the composition of the Mediterranean biota in every climatic phase (Pérès, 1985). Nevertheless, recent molecular evidence has suggested that at least some of the subtropical species currently found in the Mediterranean are not recent Holocenic invaders, but have a pre-glacial origin and survived the glacial peaks in warmer refugia within the Mediterranean (Almada et al., 2001; Domingues et al., 2007; Wilson and Eigenmann Veraguth, 2010). Molecular data indicate that the southern parts of the Mediterranean, being warmer than northern areas during the Pleistocene (Thiede, 1978), acted as refugia for sea grasses (e.g. *Posidonia oceanica*, Arnaud-Haond et al., 2007; *Cymodocea nodosa*, Alberto et al., 2008) and that the Ionian and Aegean Sea, acted in the same way for some fish species (Bahri-Sfar et al., 2000; Magoulas et al., 1996).

Marine turtles have tropical affinities and females are highly philopatric, returning to specific geographical locations to nest (Carr and Ogren, 1960; FitzSimmons et al., 1997; Meylan et al., 1990). This results in strong genetic structuring when mtDNA is considered (Bowen and Karl, 2007; Lee, 2008), allowing evolutionary and colonisation events to be traced (Garofalo et al., 2009). The loggerhead turtle (*Caretta caretta* L.) is the least thermophilic cheloniid and regularly nests in subtropical and warm temperate regions where sand temperature is higher than 24 °C for a sufficiently long period of time (Miller et al., 2003). Paleoclimatic reconstructions of sea surface temperatures indicate that loggerhead turtles could not use the Western Mediterranean even as a foraging ground due to low sea surface temperatures during the last glacial peak (summer surface temperature<17 °C; Thiede, 1978). Only the Eastern Mediterranean was warm enough to allow turtle nesting, as summer sea surface temperatures were usually higher than 22 °C (Thiede, 1978); the minimum threshold for loggerhead turtle nesting (Miller et al., 2003). Thus, in the case that *C. caretta* had already colonised the Mediterranean prior to glaciation events, these Eastern regions could have acted as refugia for loggerhead turtles through the cold phases of the Pleistocene. Nevertheless, Bowen et al. (1993a) proposed a recent Holocenic origin for loggerhead turtles currently nesting in the Mediterranean. However, their conclusion was based on the analysis of just one nesting ground from the Ionian Sea (Bay of Kyparissia), the only rookery sampled at that time. New genetic data on the Mediterranean populations have come to light since (Carreras et al., 2007; Chaieb et al., 2010; Encalada et al., 1998; Garofalo et al., 2009; Laurent et al., 1998; Saied et al., 2012; Yilmaz et al., 2011).

To track the colonisation history of the Mediterranean by loggerhead turtles and to test the possible existence of warm refugia during the cold phases we have analysed mtDNA sequences from multiple nesting grounds in the Eastern Mediterranean, including previously poorly sampled locations.

2. Material and methods

2.1. Sample collection

Samples of skin and/or muscle were taken from 168 dead hatchlings and embryos from unhatched eggs during post-hatch nest excavations of nesting grounds in the Mediterranean Sea between 2003 and 2006 (Fig. 1, Table 1). These included Libya (west of Sirte), Israel (scattered sites along the whole coastline), Lebanon (El Mansouri), Cyprus (Alagadi and Akamas) and Greece, with samples from Western Greece (Zakynthos and Lakonikos Bay) and Crete (Rethymno). Samples were stored in 95% ethanol and samples from Greece, Israel and Lebanon previously analysed by Carreras et al. (2007) were also used for this study. Independency among samples can be assumed because sampling included protocols to avoid pseudoreplication. These included female tagging and samples taken from clutches laid within a 15-day window to avoid hatchlings from the same individual turtle, as females rarely nest at intervals shorter than this period (Dutton, 1995). However, the new samples from Lebanon were collected in different years from those from Carreras et al. (2007) and hence, additional pseudoreplication tests were undertaken to ensure independency between samples. Pseudoreplication was assessed by amplifying the new samples with seven microsatellite loci (Carreras et al., 2007) and comparing them with the Lebanon samples in Carreras et al. (2007). A pairwise relatedness analysis implemented in GenAlEx v6.4 (Peakall and Smouse, 2006) was used for the comparison.

2.2. DNA extraction and amplification

DNA was extracted with the QIAamp extraction kit (QIAGEN®) and an 815 bp fragment of the mtDNA control region was amplified by polymerase chain reaction (PCR) using the primer pair LCM15382 (5′-GCTTAACCCTAAAGCATTGG-3′) and H950 (5′-GTCTCGGATTTAGGG GTTT-3′) (Abreu-Grobois et al., 2006). The analysis of longer sequences has been proven to improve the genetic resolution in *C. caretta* populations (Monzón-Argüello et al., 2010; Saied et al., 2012). The resulting fragment contains the 380 bp fragment traditionally used for population studies on this species (Carreras et al., 2006; Encalada et al., 1998; Norman et al., 1994). PCR cycling parameters were 94 °C for 5 min followed by 35 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 90 s, and a final extension period of 72 °C for 10 min. Resulting products were purified by enzymatic reaction (ExoSAP) and sequencing reactions undertaken with fluorescent dye terminators (BigDye v3.1®). All samples were sequenced in both forward and reverse directions on an ABI 3730 automated DNA Analyser (Applied Biosystems®) to confirm variable sites on both strands of DNA.

2.3. Data analysis

Alignment was conducted using BioEdit v5.0.9 (Hall, 1999) and sequences were compared to short and long haplotypes previously described for this species and compiled by the Archie Carr Center for Sea Turtle Research of the University of Florida (ACCSTR; http://accstr.ufl.edu). New haplotypes identified were named following ACCSTR standardised nomenclature and submitted to GenBank (Accession nos. JF837821–JF837824).

To understand the genetic relationships between the sampled rookeries, pairwise genetic distances (γ_{st}) were calculated by the DnaSP v5 software package (Librado and Rozas, 2009). The significance of genetic differentiation among these regions was assessed using Hudson's nearest neighbour statistics (S_{NN}) with 1000 permutations in DnaSP. Published long sequence data from Southern Italy (Calabria; Garofalo et al., 2009) and Turkey (Yilmaz et al., 2011, which includes Turkish samples from Carreras et al., 2007) were also used in the analyses. Five nesting groups were considered in Turkey as suggested by the authors' conclusions (Yilmaz et al., 2011): Dalyan, Dalaman, Western Turkey (Fethiye, Patara, Kale, Kumluca and Çirali), middle Turkey (Gazipaşa, Kizilot, Tekirova and Belek) and Eastern Turkey (Anamur, Göksu Deltasi, Alata, Kazanli, Akyatan, Ağyatan and Samandağ). Recently published data from Libya (Saied et al., 2012) were not added to our dataset to avoid pseudoreplication as samples from both datasets were collected from the same location (Sirte) within a three year window. However, genetic differentiation analyses were undertaken with both datasets separately to look for possible differences. Following Narum (2006), modified false discovery rate (FDR) was used to evaluate statistical significance instead of the sequential Bonferroni correction when analysing multiple comparisons. Haplotype diversity (h; Nei, 1987) and nucleotide diversity (π; Nei, 1987) were estimated using ARLEQUIN v3.1 (Excoffier et al., 2005) and Fu's *Fs* values for each nesting region were calculated with DnaSP.