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Philopatry in loggerhead turtles *Caretta caretta*: beyond the gender paradigm

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ABSTRACT: Marine turtles have been traditionally considered model organisms to study sex-biased behaviour and dispersal. Although female philopatry has been identified in the loggerhead turtle, with adult females returning to specific locations to nest, studies on the philopatry and breeding migrations of males remain limited. In this study we analysed 152 hatchlings using 15 microsatellite markers. Each individual came from a different nest from samples taken at 8 nesting grounds in the Mediterranean. Our results revealed the existence of 5 genetically differentiated units, mostly due to restricted gene flow for both sexes. This supports existing satellite tracking studies that suggest that mating occurs close to nesting grounds in this region. The 5 management units identified within the Mediterranean included nesting grounds from (1) Libya and Cyprus, (2) Israel, (3) Lebanon, (4) Turkey and (5) Greece. The genetic similarity between distant nesting areas (i.e. Libya and Cyprus) suggests the presence of a more complex pattern of breeding behaviour. Three possible hypotheses, that remain to be tested in future studies, could explain this result: (1) mating might take place in common foraging grounds; (2) mating could occur en route while migrating to/from the breeding grounds; or (3) recent colonisation events could connect the 2 nesting grounds. Overall, our work suggests that widespread male-mediated gene flow between loggerhead nesting grounds is likely to have been previously overstated although opportunistic breeding patterns might connect some widely separated areas.

KEY WORDS: *Caretta caretta* · Gene flow · Microsatellites · Philopatry · Nesting grounds · Population structure

INTRODUCTION

Wildlife conservation is a worldwide priority because of strong population declines in many endangered species due to anthropogenic impacts (Gray 1997). Defining management units (MUs) is of crucial importance in order to develop successful management plans for endangered species (Schwartz et al. 2007, Rees et al. 2016). Many studies have focused on population structuring and gene flow between popu-

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lations through the use of DNA markers; however the detection of genetic differentiation may depend on the populations analysed, the sample sizes used and the type and number of molecular markers (Beebee & Rowe 2008). Genetic studies of mitochondrial DNA (mtDNA), a maternally inherited marker, have revealed strong population structuring among a variety of species at different spatial scales, from honey bees *Apis mellifera* (Garnery et al. 1993) to bottlenose dolphins *Tursiops* spp. (Krützen et al. 2004), as well as at temporal scales in the case of the crab *Liocarcinus depurator* (Pascual et al. 2016). However, while mtDNA is a powerful marker for the study of population structure and phylogeographic processes, it does not take into consideration the contribution of males to the genetic structure of populations. The analysis of nuclear DNA (nDNA), representative of both female- and male-mediated gene flow, is of relevance when designing conservation and management plans, as both sexes might not behave similarly (Prugnolle & de Meeus 2002, Lawson Handley & Perrin 2007).

Sea turtles have been considered good model species to study complex population structure mediated by sex-biased dispersal (Bowen & Karl 2007). However, whilst previous research has revealed a strong female philopatry in sea turtles, with adult females returning to specific locations to nest (Miller et al. 2003), less is known about philopatry, distribution patterns and breeding migrations of males (FitzSimmons et al. 1997a). Adult turtles typically migrate from foraging grounds to breeding areas (Limpus 1993, Frick et al. 2000) and, after mating, males return to their foraging grounds while females remain to nest on sandy beaches (Schofield et al. 2010, Arendt et al. 2012). These general patterns have been widely studied through long-term tag-recovery, telemetry and stable isotope analyses (Godley et al. 2010). However, research has been traditionally skewed to females as they are easier to sample while laying eggs in monitored nesting beaches. It is now globally accepted that males follow similar migration patterns as females (Hatase et al. 2002, Godley et al. 2008), although they tend to migrate to foraging grounds that are closer to the breeding grounds (Van Dam et al. 2008, Arendt et al. 2012), and that males show strong fidelity to the same breeding sites (Casale et al. 2013, Hays et al. 2014). However, timing and frequency of male migrations may vary among species and populations (Hays et al. 2010). Moreover, male satellite tracking has shown some cases of fidelity to the same breeding ground but also the ability of males to frequent multiple breeding grounds within the same season (Schofield et al. 2010, 2013). Whilst previous studies have shown that mating regularly occurs close to nesting beaches (Godley et al. 2002, Schofield et al. 2017), it is still unclear whether mating also takes place in foraging grounds or en route to and/or from nesting beaches.

Different sea turtle nesting populations with overlapping habitats might interbreed, increasing gene flow and significantly reducing genetic differentiation between populations for bi-parentally inherited markers. Lower genetic structuring has been observed at nDNA level compared to mtDNA among nesting areas (Birky et al. 1989, Jensen et al. 2013). This has been generally considered to be the result of male-mediated gene flow, described for green turtles *Chelonia mydas* (Karl et al. 1992, FitzSimmons 1997a, Roberts et al. 2004) and also for loggerhead turtles *Caretta caretta* nesting in the northwestern Atlantic (Bowen et al. 2005). However, fine-scale female and male loggerhead philopatry was detected in Cape Verde, with nDNA showing directional male-mediated gene flow (Stiebens et al. 2013).

The loggerhead turtle hosts an independent regional management unit (RMU) (Wallace et al. 2010) in the Mediterranean Sea, genetically separated from that in the Atlantic Ocean (Wallace et al. 2010, Carreras et al. 2011). Regular nesting only occurs in the eastern Mediterranean (Margaritoulis et al. 2003, Casale & Margaritoulis 2010), although some sporadic nesting has been reported in the western Mediterranean (Carreras et al. 2018). Genetic studies have revealed that nesting grounds in the eastern Mediterranean exhibit deep mtDNA genetic structuring (Laurent et al. 1998, Yilmaz et al. 2011, Saied et al. 2012), derived from a combination of female philopatry, isolation by distance, sequential colonisation and the use of glacial refugia during the Pleistocene (Clusa et al. 2013). Several MUs have been described within the Mediterranean RMU based on the frequency of mtDNA haplotypes (Shamblin et al. 2014). Relevant population structure based on nDNA among Mediterranean nesting beaches was first reported along the Turkish coast using randomly amplified polymorphic DNA (RAPD) markers (Schroth et al. 1996). Significant structure among some nesting grounds was revealed by Carreras et al. (2007) in a study comprising several eastern Mediterranean nesting grounds and using 7 microsatellite markers. In contrast, 2 other studies within the Mediterranean Sea identified poor genetic differentiation, attributed to male-mediated gene flow across nesting grounds (Yilmaz et al. 2011) or foraging grounds (Garofalo et al. 2013) based on 6 and 4 microsatellite loci, respectively. Differences among studies

could be partly due to sampling size effects as well as to the reduced number of markers used (Dutton et al. 2013) and thus both the number of MUs in the eastern Mediterranean and the male-mediated connectivity hypothesis remain controversial.

The primary aim of this work is to establish the influence of female- and male-mediated gene flow in structuring genetic differentiation among Mediterranean loggerhead nesting grounds. In order to do so, we sampled 8 Mediterranean nesting grounds, from 6 countries included in a study of large nesting sites by Almpanidou et al. (2016), to determine with nuclear markers whether male-mediated gene flow exists across this broad region. The aim of the study was to (1) assess the genetic structuring in Mediterranean loggerhead nesting grounds using 15 microsatellite loci, (2) redefine MUs in the Mediterranean Sea and (3) contrast the results on genetic structuring based on nuclear and mitochondrial DNA to unveil the breeding behaviour of males and females of this endangered species.

MATERIALS AND METHODS

Sampling locations

Samples of skin and/or muscle were taken from 152 dead loggerhead turtle hatchlings, each from a different nest. The samples were collected from 8 nesting grounds in the Mediterranean Sea (Fig. 1, Table 1), which have variable numbers of nests per season (Casale & Margaritoulis 2010) and are considered large nesting areas (Almpanidou et al. 2016). Samples were obtained during 2003 to 2006 (Table 1 and Supplement 1 at www.int-res.com/articles/suppl/m588p201_supp.xlsx). The same samples were used in a previous study with a partial sequence of the mtDNA control region (Clusa et al. 2013), thus allowing comparison between nuclear and mitochondrial markers. Samples were taken from nesting grounds at west of Sirte, Libya (LIB), scattered sites along the coastline from Haifa to Natanya, Israel (ISR), El Mansouri, Lebanon (LEB), Fethiye, Turkey (WTU), Akamas in Chrysochou Bay, Cyprus (CYP), and 3 sites in Greece: Rethymno on the Island of Crete (CRE), Lakonikos Bay (LAK) and Zakynthos (ZAK). Abbreviations are the same as those used by Clusa et al. (2013). Samples were collected from all the above-mentioned nesting beaches except those in Israel. The number of loggerhead females nesting in Israel is small and scattered along the region as indicated in previous studies (Table 5 in Levy 2010).

Nests were excavated after hatchling emergence and samples were collected from 1 dead hatchling per nest and stored in 95% ethanol. Loggerhead females may re-nest within the same reproductive season but rarely within a 15 d period (Miller et al. 2003). Hence, samples were taken from clutches laid within a 15 d window to avoid pseudoreplication (Dutton 1996). However, internesting intervals vary with temperature (Hays et al. 2002), and shorter periods of collection should be considered in order to fully rule out pseudoreplication.

DNA extraction and microsatellite loci scoring

DNA was extracted with the QIAamp extraction kit (QIAGEN®) and 15 microsatellite loci previously used for loggerhead turtle studies were amplified: Cc117, Cm72 and Cm84 (FitzSimmons et al. 1995); Ccar176 (Moore & Ball 2002; modified by Carreras et al. 2007); Cc7 and Cc141 (Bowen et al. 2005); and Cc2, Cc10, Cc13, Cc16, Cc17, Cc22, Cc25, Cc28 and Cc30 (Monzón-Argüello et al. 2008). One primer for each marker was fluorescently labelled with 6-FAM, NED, PET or VIC. The protocol set by Carreras et al. (2007) was used to amplify Cc117, Cm72, Cm84, Ccar176 Cc7 and Cc141. Four PCR multiplex reac-

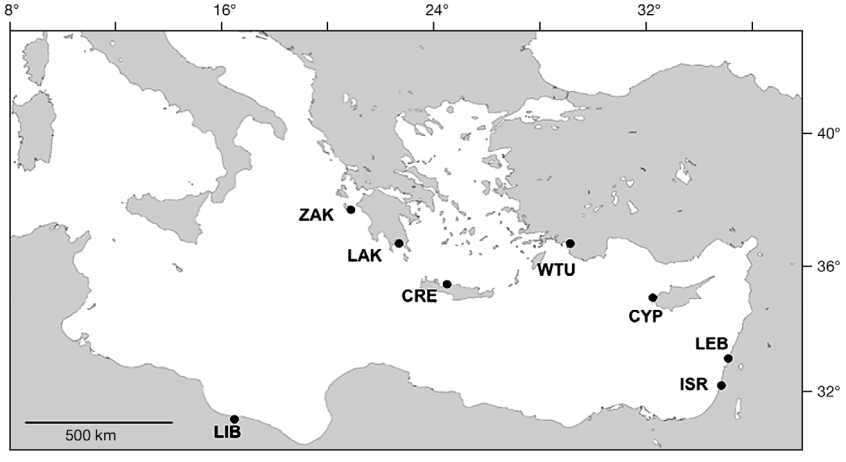

Fig. 1. Locations of sampled loggerhead turtle *Caretta caretta* nesting grounds in the Mediterranean. LIB: Sirte, Libya; ISR: scattered sites along the coastline from Haifa to Natanya, Israel; LEB: El Mansouri, Lebanon; CYP: Akamas, Cyprus; WTU: Fethiye, Turkey; CRE: Rethymno, Greece; LAK: Lakonikos, Greece; ZAK: Zakynthos, Greece

Table 1. Loggerhead turtle nesting grounds in the Mediterranean analysed in this study. The abbreviations used are the same as in Clusa et al. (2013). Data on the number of nests per season at each site are adapted from Casale & Margaritoulis (2010). Samples of skin and/or muscle from dead loggerhead turtle hatchlings were collected from the named nesting beach in all cases except Israel, where the samples were obtained from different nesting beaches between Haifa and Natanya. n: number of analysed individuals; *Ar*: allelic richness; H_o: observed heterozygosity; H_e: expected heterozygosity. Data for *Ar*, H_o and H_e are mean ± SE

Location	Acronym	Nests per season	Sampling year	n	*Ar*	H_o	H_e
Sirte, Libya	LIB	343–359	2005–06	27	4.678 ± 0.336	0.649 ± 0.054	0.660 ± 0.045
Haifa–Natanya, Israel	ISR	12–80	2001–03	19	4.826 ± 0.379	0.649 ± 0.059	0.681 ± 0.050
El Mansouri, Lebanon	LEB	40–122	2004–06	19	4.732 ± 0.336	0.622 ± 0.039	0.669 ± 0.038
Akamas, Cyprus	CYP	123–356	2005	21	4.630 ± 0.363	0.629 ± 0.059	0.668 ± 0.046
Fethiye, Turkey	WTU	169–523	2003	17	4.002 ± 0.268	0.619 ± 0.054	0.619 ± 0.042
Rethymno, Greece	CRE	166–516	2003	18	4.194 ± 0.358	0.591 ± 0.058	0.623 ± 0.049
Lakonikos, Greece	LAK	107–288	2003	18	4.059 ± 0.337	0.594 ± 0.057	0.596 ± 0.047
Zakynthos, Greece	ZAK	833–2018	2003	13	4.072 ± 0.301	0.557 ± 0.062	0.597 ± 0.052

tions designed by Monzón-Argüello et al. (2008) were used to amplify the remaining 9 new microsatellite loci, following the author's protocol. When multiplex PCR reactions failed to amplify, single PCRs were performed for the failed microsatellite amplifications following the protocol in Carreras et al. (2007). Fragment lengths were estimated on an ABI 3730 automated sequencer at the Scientific-Technical Services at the University of Barcelona, with GeneScan 500 LIZ (Applied Biosystems) as an internal size standard. Allele sizes were assigned with GeneMapper v.3.5 (Applied Biosystems).

Statistical analyses

The mean observed (H_o) and expected (H_e) heterozygosities were calculated for each nesting ground using GenAlEx v.6.5 (Peakall & Smouse 2012). The mean allelic richness (*Ar*) was estimated with the program Fstat v.2.9.3 (Goudet 1995). Differences in diversity among nesting grounds were evaluated with a Friedman ANOVA test in STATISTICA v.8 (www.statsoft.com). Deviations from Hardy-Weinberg equilibrium and linkage disequilibrium between loci were assessed with Genepop v.4.1 (Rousset 2008) and the presence of null alleles was inferred with FreeNA (Chapuis & Estoup 2007). The shortest distances along the coastline from each nesting grounds to LIB (Fig. S1 in Supplement 2 at www.int-res.com/articles/suppl/m588p201_supp.pdf), the oldest Mediterranean unit according to mtDNA (Clusa et al. 2013), were calculated using the ArcGIS v.9 software (ESRI 2011). Coastal and Euclidean geographic distances (Table S1 in Supplement 2) among all nesting grounds were calculated with the same software. Distances were measured among sampled beaches in all cases but Israel, where the mid-point distance of all sampled nests was used. The coastal distances between island locations and mainland locations were chosen as the shortest distance between the 2 sites. Linear regressions of *Ar*, H_o and H_e with the shortest distances from each nesting ground to LIB were calculated with STATISTICA.

Pairwise genetic distances (F_{ST}) among nesting grounds were calculated with Genepop v.4.1 and the significance of differentiation (*G*-test) assessed by Markov chain Monte Carlo (MCMC) randomisation. Pairwise genetic distances were also calculated using D_{ST} (Jost 2008) with DEMEtics (Gerlach et al. 2010) and 10 000 iterations performed to calculate the significance of pairwise differences. Congruence between F_{ST} and D_{ST} distance measurements was analysed through a Mantel test with GenAlEx v.6.5. The congruence between pairwise population genetic differentiation found with nDNA (this study) and that published for mtDNA for the same samples (γ_{ST}) (Clusa et al. 2013) was assayed using the same approach. Principal coordinate analyses (PCoA) as implemented in GenAlEx v6.5 were used to plot D_{ST} and γ_{ST} pairwise distances between nesting grounds for comparison among nDNA and mtDNA markers, respectively. The modified false discovery rate (FDR) was used to evaluate statistical significance when analysing multiple comparisons (Narum 2006). The evolutionary relationships among nesting grounds were reconstructed with a phylogenetic tree based on D_A genetic distance (Nei et al. 1983) using POPTREEW (Takezaki et al. 2014).

Isolation by distance, comparing genetic and geographic pairwise distances, was assessed through Mantel tests. Furthermore, we performed a partial

Mantel test with the Euclidean geographic distance as a covariate to remove spurious correlations due to population subdivision (Meirmans 2012). Mantel and partial Mantel correlation coefficients (r) were estimated using the zt software (Bonnet & Van de Peer 2002), with 10 000 permutations to evaluate their significance.

The most likely number of populations (K) within the area was inferred with STRUCTURE v.2.3.4 (Pritchard et al. 2000), a Bayesian algorithm for clustering. The parameter K was allowed to vary between 1 and 8 (the maximum number of nesting grounds analysed). For each K, 20 runs and 100 000 MCMC iterations were simulated with a preset 10 000 iteration burn-in. The best K was obtained with the mean likelihood of the 20 runs for each simulated number of populations, its standard deviation and the ad hoc statistic ΔK (Evanno et al. 2005) in STRUCTURE HARVESTER (Earl & vonHoldt 2012). The 20 runs for the best selected K were combined with CLUMPP v.1.1.2 (Jakobsson & Rosenberg 2007) and the result was represented with DISTRUCT v.1.1 (Rosenberg 2004).

A spatially explicit Bayesian approach was undertaken with Geneland v.4.0.3 (Guillot et al. 2005) to determine the number of genetically differentiated clusters in the study area based on genetic and geographic information. The parameter K was allowed to vary between 1 and 8, and 20 runs and 100 000 MCMC iterations with a preset 200 iteration burn-in were set to calculate K. Finally, we performed a discriminant analysis of principal components (DAPC), a multivariate method to identify genetic clustering (Jombart et al. 2010), using the function *dapc* implemented in the *adegenet* package in R (www.r-project.org). We retained 50 principal components as input of the discriminant analysis. The variables were centred but not scaled and explained 93% of the variance.

The existence of recent genetic bottlenecks was tested by the Wilcoxon sign-rank test with 100 000 iterations implemented in BOTTLENECK v.1.2.02 (Piry et al. 1999) under the assumption of a 2-phase microsatellite mutation model, with the proportion of multi-step mutations set at 57% and the variance of the mean size of multi-step mutations set at 22 as used for green turtles (FitzSimmons 1998, Peery et al. 2012). Family relatedness within nesting grounds was assessed with GenAlEx v.6.5 by the algorithm of Lynch and Ritland (LRM) (Lynch & Ritland 1999) and 95% confidence intervals were estimated using 999 permutations. Differences in relatedness between nesting grounds were evaluated by a Kruskal-Wallis test in STATISTICA v.8.

RESULTS

The 152 samples were successfully amplified and the mean number of alleles per microsatellite locus ranged from 2.75 (Cm72) to 9.5 (Cc7) (see Table S2 in Supplement 2). The independence of loci was assumed as no linkage disequilibrium was found between loci pairs (χ^2: $p > 0.050$ in all cases). However, marker Cc25 was excluded from further analyses since it departed from Hardy-Weinberg equilibrium (χ^2: FDR $p < 0.014$) and presented a high frequency of null alleles in the majority of locations (0.153 ± 0.08, mean ± SD), as inferred by FreeNA. Heterozygote proportions (Table 1) were not statistically different between nesting grounds (Friedman ANOVA: χ^2 = 11.847, $p = 0.106$), but differences were highly significant in expected heterozygosity (Friedman ANOVA: $\chi^2 = 23.157$, $p = 0.002$) and allelic richness (Friedman ANOVA: $\chi^2 = 21.356$, $p = 0.003$). The 3 diversity estimators in Table 1 (Ar, H_o and H_e) were negatively and significantly correlated to the minimum distance from LIB following the coastline (Fig. 2). Accordingly, the nesting grounds further away from LIB, i.e. LAK and ZAK, showed the lowest diversity values (Table 1). This was not biased by a few loci as values decreased from LIB to nesting grounds from Greece in >65% of the analysed loci in all 3 diversity indices (data not shown). There was evidence of recent genetic bottleneck events in CYP and WTU, as indicated by the Wilcoxon sign-rank test (Table S3 in Supplement 2).

Pairwise genetic distances between nesting grounds based on F_{ST} and D_{ST} (Table 2) were strongly correlated (Mantel test: $R^2 = 0.943$, $p < 0.001$). Significant F_{ST} and D_{ST} genetic differences were found in ca. 80% of the pairwise comparisons between all nesting grounds except between the Greek nesting grounds (CRE, LAK and ZAK) and between LIB, LEB and CYP. The PCoA plot based on D_{ST} pairwise values between locations (Fig. 3) explained 81% of the variation with the first 2 axes. The first axis, explaining 63% of the total variation, separated northern (WTU, CRE, LAK and ZAK) and southern (LIB, ISR, LEB and CYP) nesting grounds and was positively and significantly correlated to all diversity measures (H_e: $R^2 = 0.901$, $p < 0.001$; H_o: $R^2 = 0.869$, $p < 0.001$; Ar: R^2 = 0.793, $p < 0.001$). The same plot was obtained when using F_{ST}, explaining 83% of the variation with the first 2 axes, with the first also being positively and significantly correlated to all diversity measures (data not shown). Two genetically differentiated clusters were identified by STRUCTURE (K = 2). When considering 2 groups, the southern nesting grounds

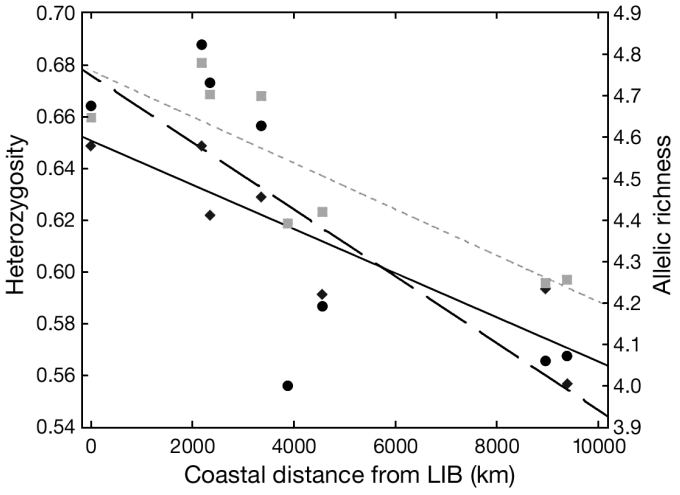

Fig. 2. Linear regressions between (◆) mean observed heterozygosity (H_o) (R^2 = 0.795, p = 0.003), (■) mean expected heterozygosity (H_e) (R^2 = 0.736, p = 0.006), and (●) allelic richness (Ar) (R^2 = 0.584, p = 0.027) from dead loggerhead turtle hatchlings at each nesting ground and the shortest distance (km) along the coastline from the nesting site at Sirte, Libya (LIB). Linear regression using H_o values (dashed grey line), H_e values (solid line), and Ar values (dashed black line)

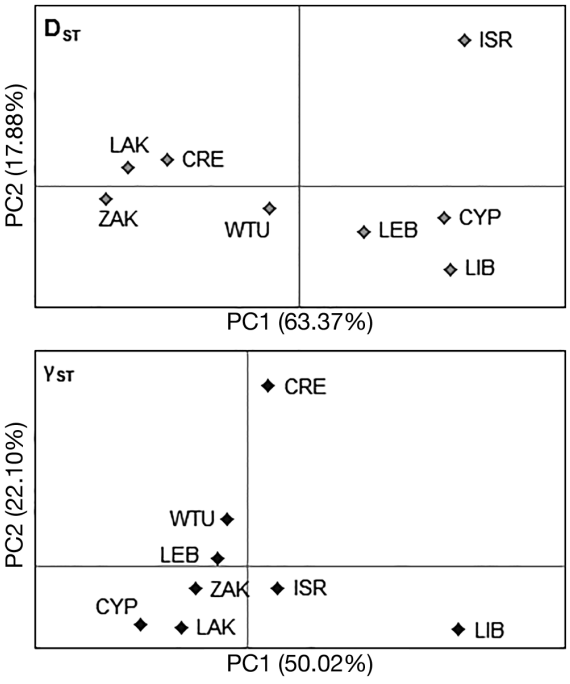

Fig. 3. Results of principal coordinate analyses based on pairwise genetic distances between loggerhead turtle Mediterranean nesting grounds for nDNA (D_{ST}) and mtDNA (γ_{ST}). See Fig. 1 for locations of nesting grounds and key to abbreviations

were separated from the northern nesting grounds (Fig. 4). The grouping of populations in a northern and southern subgroup was further supported by the phylogenetic neighbour-joining tree with high bootstrap value (Fig. S2 in Supplement 2). Differences in relatedness between individuals within populations were also detected among nesting grounds (Kruskal-Wallis: χ^2 = 14.162, p = 0.048), with mean pairwise relatedness values within nesting grounds being higher in the northern cluster (Fig. S3 in Supplement 2).

A finer-scale sub-structuring was unveiled with Geneland based on genetic and geographic information. With this approximation 5 clusters were identified (K = 5; Fig. 5a): (1) LIB and CYP, (2) ISR, (3) LEB, (4) WTU and (5) the 3 Greek nesting grounds CRE, LAK and ZAK. The first component of the DAPC also supported the differentiation of the southern and northern groups (Fig. 5b). The 5 groups identified by Geneland could also be differentiated by the first 2 axes, with ISR being separated by the second axis and WTU and LEB placed in an intermediate position.

Table 2. Pairwise genetic distances between Mediterranean nesting populations of loggerhead turtles based on genotyping dead hatchlings from different nests. F_{ST} values are below the diagonal and D_{ST} values above diagonal. **Bold** type indicates a significant correlation after false discovery rate correction for a threshold of α = 0.05 (p < 0.013). See Fig. 1 and Table 1 for locations of nesting grounds and key to abbreviations

	LIB	ISR	LEB	CYP	WTU	CRE	LAK	ZAK
LIB		**0.061**	0.008	0.019	**0.061**	**0.088**	**0.095**	**0.101**
ISR	**0.015**		**0.053**	**0.043**	**0.080**	**0.086**	**0.107**	**0.138**
LEB	0.002	**0.016**		0.016	0.028	0.031	**0.044**	**0.065**
CYP	0.007	**0.017**	0.005		0.036	**0.062**	**0.108**	**0.091**
WTU	**0.017**	**0.028**	**0.006**	**0.010**		0.033	0.021	0.044
CRE	**0.024**	**0.026**	**0.009**	**0.022**	**0.011**		−0.001	−0.017
LAK	**0.033**	**0.039**	**0.016**	**0.044**	**0.014**	−0.002		−0.007
ZAK	**0.035**	**0.045**	**0.021**	**0.035**	**0.022**	−0.011	−0.004	

Significant patterns of isolation by distance were shown by the Mantel test relating pairwise genetic distances (F_{ST}) and coastal distances (r = 0.715, p = 0.002). Coastal pairwise distances ranged between 160 and 9400 km and Euclidean pairwise distances, these being the shortest geographic distances among nesting grounds, between 110 and 1630 km. The correlation between genetic and Euclidean pairwise distances among nesting grounds was much smaller (r = 0.369, p = 0.049). The partial Mantel test carried out to discard spurious correlation due to population subdivision was highly significant (r = 0.659, p = 0.001). Similar values

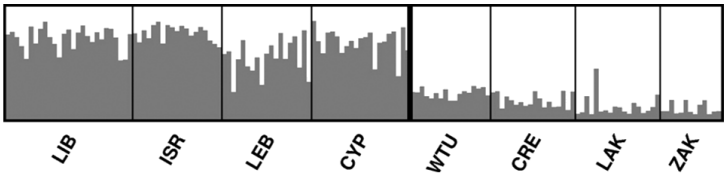

Fig. 4. *Caretta caretta.* Assignment probabilities of each individual loggerhead turtle hatchling to each genetically differentiated cluster identified by STRUCTURE ($K = 2$). Each bar shows the probability of an individual to belong to the southern cluster (grey) or to the northern cluster (white). See Fig. 1 for locations of sampling sites and key to abbreviations

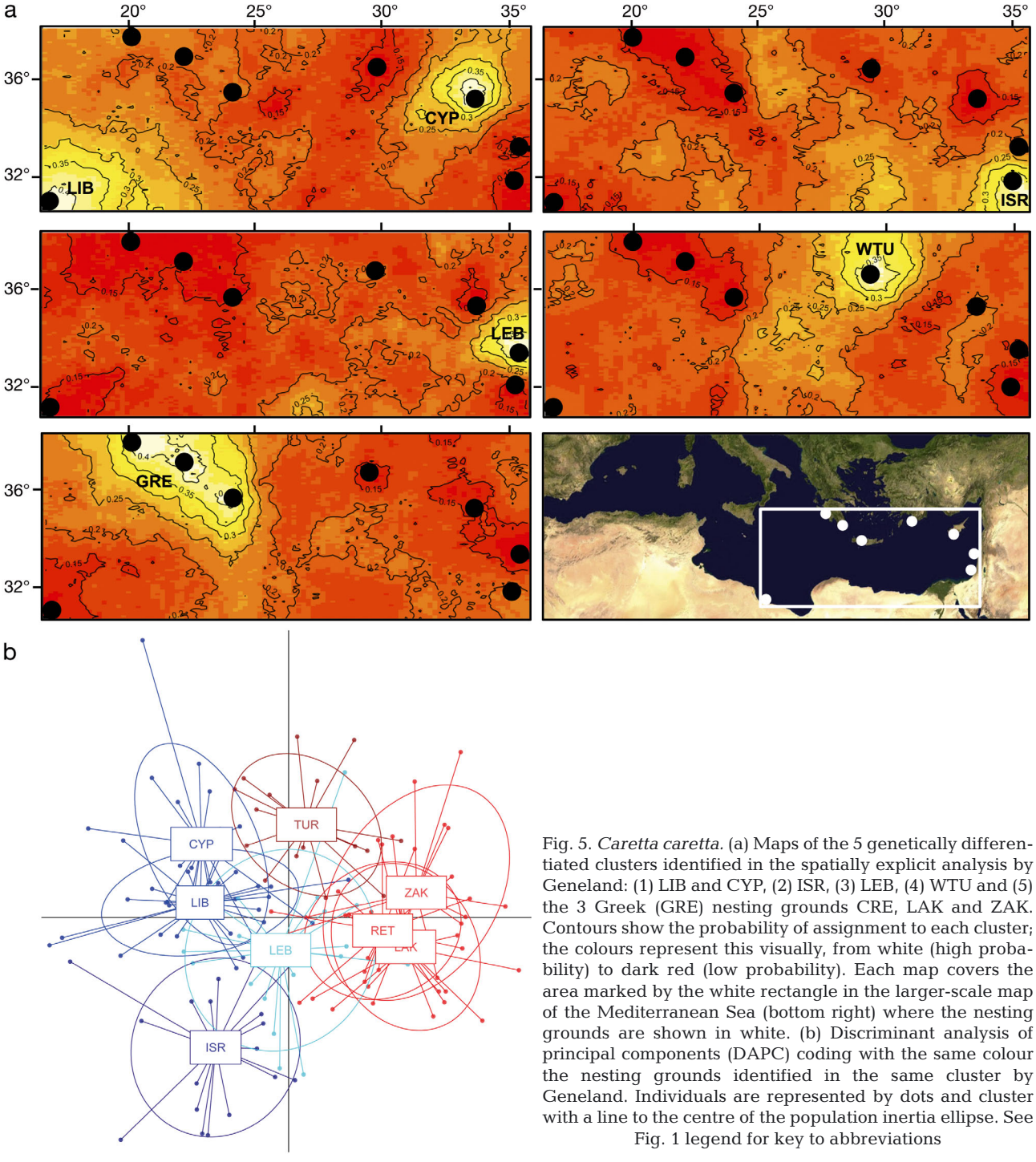

Fig. 5. *Caretta caretta.* (a) Maps of the 5 genetically differentiated clusters identified in the spatially explicit analysis by Geneland: (1) LIB and CYP, (2) ISR, (3) LEB, (4) WTU and (5) the 3 Greek (GRE) nesting grounds CRE, LAK and ZAK. Contours show the probability of assignment to each cluster; the colours represent this visually, from white (high probability) to dark red (low probability). Each map covers the area marked by the white rectangle in the larger-scale map of the Mediterranean Sea (bottom right) where the nesting grounds are shown in white. (b) Discriminant analysis of principal components (DAPC) coding with the same colour the nesting grounds identified in the same cluster by Geneland. Individuals are represented by dots and cluster with a line to the centre of the population inertia ellipse. See Fig. 1 legend for key to abbreviations

Table 3. Review of published marine turtle studies assessing genetic differentiation among rookeries with mtDNA and different numbers of microsatellite loci (nDNA). For each study total pairwise comparisons are classified as showing or not showing significant differences using mtDNA and nDNA. Male-mediated gene flow (MM) is represented by the proportion of pairwise comparisons that do not support male philopatry (non-significant differences using nDNA) when females are philopatric (significant differences using mtDNA). N: mean number of individuals analysed with mtDNA and nDNA per rookery; Msat: number of microsatellites; PC: total number of pairwise comparisons; sig.: significant difference; ns: no significant difference; na: no value of MM is available due to the lack of differentiation found using mtDNA

	N	Msat	PC	mtDNA sig.		mtDNA ns		MM	Source
				nDNA ns	nDNA sig.	nDNA ns	nDNA sig.		
Caretta caretta									
W Atlantic	51.3	5	36	18	11	5	2	0.62	Bowen et al. (2005)
Pacific	56.8	5	10	5	0	3	2	1.00	Watanabe et al. (2011)
Turkey	51.2	6	10	6	3	1	0	0.67	Yilmaz et al. (2011)
Mediterranean	16.1	7	21	0	2	12	7	0.00	Carreras et al. (2007)
Atlantic	22.2	8	6	0	3	3	0	0.00	Stiebens et al. (2013)
E Atlantic	42.7	12	3	0	0	3	0	na	Monzón-Argüello et al. (2010)
Mediterranean	19	14	28	4	14	2	8	0.22	Present study
Chelonia mydas									
Pacific	30.7	3	6	0	0	6	0	na	Chassin-Noria et al. (2004)
Pacific/Indian	12.9	4	15	10	2	3	0	0.83	Roberts et al. (2004)
Atlantic/Mediterranean	27.4	4	28	20	5	3	0	0.80	Roberts et al. (2004)
Pacific	68.5	4	6	1	5	0	0	0.17	FitzSimmons et al. (1997b)
Pacific	22.3	4	3	2	0	1	0	1.00	Nishizawa et al. (2011)
Mediterranean	42.7	6	14	1	0	5	8	1.00	Bagda et al. (2012)
Pacific	61.6	8	10	0	10	0	0	0.00	Roden et al. (2013)
Atlantic	50.2	15	15	0	14	0	1	0.00	Naro-Maciel et al. (2014)
Dermochelys coriacea									
Atlantic	157.4	17	36	0	34	0	2	0.00	Dutton et al. (2013)

were obtained when D_{ST} pairwise distances were used in the comparison (data not shown). No correlation was found between pairwise genetic distances analysed with nDNA (this study) and previously published genetic distances based on long D-loop mtDNA sequences (Clusa et al. 2013) using the same samples (F_{ST}: r = −0.112, p = 0.323; D_{ST}: r = −0.033, p = 0.492). The PCoA plot based on mtDNA (γ_{ST}; Fig. 3) explained 72% of the observed variation, with Libya and Crete being the most differentiated nesting grounds, obtaining a different structure in comparison to the plot based on microsatellite pairwise distances.

DISCUSSION

We detected strong philopatry for both female and male loggerhead turtles in the Mediterranean Sea based on nDNA (15 microsatellites) although complex breeding patterns may coexist. Our results revealed the existence of 5 genetically differentiated units among Mediterranean loggerhead nesting grounds, mostly as a result of isolation by distance due to restricted gene flow following the coastline. Thus, the detected levels of genetic isolation among nesting grounds suggest that mating occurs in nearby breeding grounds, in agreement with mating activity detected with direct surveys (Schofield et al. 2017). Nonetheless, genetic similarity detected between distant nesting grounds indicates the existence of long-distance gene flow.

Many diverse species, such as sperm whales and sharks, may present philopatry only in females, with strong structuring in mtDNA, but not in nDNA, due to male-mediated gene flow (Lyrholm et al. 1999, Pardini et al. 2001). However, the results here suggest philopatry of both sexes in most of the loggerhead turtle nesting grounds analysed. Philopatry has been described as a successful strategy for female sea turtles to ensure suitability of nesting beaches, but also as a convenient behaviour for males since philopatry increases the chances of finding available females to mate with (Schroeder et al. 2003). Widespread male-mediated gene flow among nesting grounds (Karl et al. 1992, Bowen et al. 2005) should no longer be considered the general pattern for sea turtles, since male philopatry to breeding grounds has now been reported for various different species and populations (FitzSimmons et al. 1997a, Dutton et al. 2013, Naro-Maciel et al. 2014, this study).

Significant genetic structuring among populations based on nDNA could have been neglected in most previous marine turtle studies, since the ability to accurately assess the magnitude of differentiation seems to be linked to the number of markers, sample sizes and populations used (see Table 3). For instance, in green turtles (Roberts et al. 2004, Roden et al. 2013), population differentiation increased significantly when increasing the number of markers and samples used. This is further supported in Mediterranean loggerhead nesting grounds, where the level of differentiation between most pairwise population comparisons increased when analysing the same individuals from a previous study (Carreras et al. 2007) but amplifying for a larger number of markers (this study, see Table S4 in Supplement 2). However, no differentiation was observed among the largest Greek nesting grounds despite increasing the number of markers. This confirms that there is high gene flow among ZAK, LAK and CRE (see below). In regards to Atlantic loggerhead populations, no structuring has been detected to date (Bowen et al. 2005), suggesting that widespread male-mediated gene flow might occur among those populations. Nonetheless, this result was based on only 5 microsatellite loci, which might have underestimated the level of population differentiation as noted above. Thus, more markers would be necessary to prove or disprove widespread male-mediated gene flow in Atlantic loggerhead populations since the number of nuclear markers, together with the sequence length of mitochondrial markers, are key factors for detection of possible differentiation among populations, as shown in our study.

The differentiation detected with nDNA in the Mediterranean nesting grounds was not congruent with the units detected with mtDNA by Clusa et al. (2013), as seen in Fig. 3, even when using the same individuals. Discrepancies may arise from behavioural differences between males and females but also may be due to the higher power to detect fine-scale differentiation that multiple microsatellites have in comparison to single mtDNA markers. In the present study, we have identified 5 genetically differentiated units of *Caretta caretta* that should be considered as MUs within the Mediterranean RMU: (1) LIB-CYP, (2) ISR, (3) LEB, (4) WTU and (5) CRE-LAK-ZAK.

Two close neighbouring nesting grounds, ISR and LEB, were significantly differentiated with nDNA but not with mtDNA despite a lack of an oceanographic barrier between them. This genetic differentiation could be attributed to strong philopatry of both sexes and the lower resolution of mtDNA analyses. Alternatively, genetic drift could also explain the observed genetic differentiation between them. Extensive turtle exploitation was reported in the eastern Mediterranean Sea during the 1920s (Sella 1982). Even if the decrease in population sizes happened too recently to detect a bottleneck (Peery et al. 2012), allele frequency changes could have arisen from it, masking male-mediated gene flow. Temporal studies would be needed to test these hypotheses.

While CRE was statistically differentiated from western Greece with mtDNA, presenting some exclusive haplotypes (Clusa et al. 2013), nDNA analyses clustered all Greek nesting grounds together (Carreras et al. 2007, this study), showing that male-mediated gene flow exists between these neighbouring nesting grounds. This is supported by previous tracking studies (Casale et al. 2013) that described the movement of a male individual between the breeding grounds of ZAK and LAK. Females nesting in Greece migrate to northern foraging grounds to feed in the highly productive Adriatic Sea (Cardona et al. 2014) but also southwards to foraging grounds in North Africa and in particular the Gulf of Gabes (Schofield et al. 2013). Even if males from the region seem to use the same foraging grounds as females, and use more than one breeding area within a single season (Casale et al. 2013), a large number of males have been found to remain in close proximity to their breeding sites in Greek waters (Schofield et al. 2013, Rees et al. 2017).

WTU was significantly differentiated from the other groups of Mediterranean nesting grounds both using mtDNA (Clusa et al. 2013) and nDNA (Carreras et al. 2007, this study). However, the differentiation values found here were on average higher than in previous studies, most likely due to the larger number of nuclear markers. The strong differentiation of this population suggests that not only females but also males show philopatric mating behaviour, similar to the other Levantine nesting beaches. Moreover, the Wilcoxon sign-rank test identified a recent bottleneck in the Turkish population, consistent with a small effective population size, low diversity and high differentiation. This is in agreement with previous studies detecting further sub-structuring with a smaller number of microsatellite loci over different nesting beaches within Turkey (Yilmaz et al. 2011), as well as isolation by distance with RAPD markers (Schroth et al. 1996). Unfortunately, only samples from western Turkey were available for the present study and, hence, internal sub-structuring of Turkish nesting populations could not be re-assessed.

Mating close to the nesting beaches, combined with both male and female philopatry, likely explains the genetic structuring reported here at most breeding grounds. However, the lack of genetic differentiation found between CYP and LIB suggests a more complex pattern of breeding behaviour. Three possible hypotheses could explain this result: (1) some mating may take place in shared foraging grounds; (2) mating could take place en route while migrating; or (3) a recent colonization of CYP from LIB might have taken place. Female turtles of different species are known to be receptive for limited periods of time (Wood & Wood 1980), most likely during or after the migration to the nesting areas (Wibbels et al. 1990). However, a significant decrease of serum E_2, which is suggested to influence mating behaviour, has been reported in *C. caretta* to start 2 wk prior to migration from feeding grounds (Wibbels et al. 1990), indicating that some females could also be receptive prior to migration. Female turtles can store sperm in the upper oviduct (Gist & Jones 1989, Pearse & Avise 2001) until eggs are laid, but the quality and viability of sperm may decrease with time (FitzSimmons 1998). It has been hypothesised that females might upgrade their offspring by mating again with secondary males closer to oviposition (Moore & Ball 2002). The coast off Tunisia and Libya is a major foraging ground for adult loggerhead and green turtles from all the Mediterranean nesting grounds (Zbinden et al. 2011, Casale et al. 2013, Patel et al. 2015, Stokes et al. 2015), and females from Cyprus are known to use the Gulf of Gabes foraging area (Schofield et al. 2013). The inferred origin of individuals found in sporadic nesting events indicated that females could be receptive and that mating occurs at foraging grounds (Carreras et al. 2018). Under this scenario, gene flow would be influenced by the frequency of individuals from the different breeding grounds feeding in these common areas. To test this hypothesis, the frequency of individuals feeding in this area should be assessed combining tracking and stable isotopes (Bradshaw et al. 2017) together with high throughput markers shown to detect finer-scale differentiation in other species (Carreras et al. 2017).

Alternatively, mating could be occurring en route while migrating, as boat surveys have demonstrated (Lee et al. 2007), or when males frequent alternative nesting areas before and after mating at the primary breeding grounds (Casale et al. 2013, Schofield et al. 2013). An existing coastal corridor between the foraging ground in the Gulf of Gabes (off Libya) and Cyprus has been defined for young juveniles (Casale & Mariani 2014) and adults (Snape et al. 2016). Under this scenario, CYP females could be mating en route with LIB males when starting migration towards their nesting areas or CYP males mate with LIB females when migrating between feeding and breeding grounds following the African north coast. Thus, individuals from CYP would have more opportunities for breeding with those from LIB, as they travel along the Libyan nesting areas (Snape et al. 2016) while Greek individuals do not (Schofield et al. 2013). Moreover, where males abound off the nesting beaches, secondary mating might eventually result in the loss of any relevant contribution of the males' sperm stored in more distant areas. Thus, estimating breeding operational sex ratios in different areas, as successfully done using drones (Schofield et al. 2017), and the use of genome-wide markers to infer gene flow directionality could contribute in testing the different hypotheses. Finally, a recent colonisation of CYP from LIB could also explain the higher similarity between these 2 sites. This could be supported by the low haplotype diversity found in CYP, with the most common haplotype occurring at high frequency and only a singleton detected deriving from a mutational step (Clusa et al. 2013), and the bottleneck detected in the present study. All the possible scenarios remain hypotheses to be tested in future studies.

In conclusion, nDNA studies are crucial for defining genetically distinct units in the Mediterranean Sea which arise from a complex mating scenario with the existence of strong philopatry of both sexes to nesting grounds, male-mediated gene flow among neighbouring nesting grounds and similarity between long-distance populations. Consequently, future regional management plans should consider this substructuring to ensure the conservation of the genetic diversity found among Mediterranean populations.

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