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The genetic structure of the loggerhead sea turtle (*Caretta caretta*) in the Mediterranean as revealed by nuclear and mitochondrial DNA and its conservation implications

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Abstract The population genetic structure of the loggerhead sea turtle (*Caretta caretta*) nesting in the eastern Mediterranean was assessed by sequencing a fragment of the control region of the mitochondrial DNA (n = 190) and seven microsatellites (n = 112). The two types of markers revealed genetic structuring (mtDNA: $\gamma_{st} = 0.212$, $P < 0.001$; nDNA $F_{st} = 0.006$, $P < 0.001$), thus indicating that both females and males are philopatric and that gene flow between populations is restricted. Mitochondrial DNA data indicate that the female populations nesting on the islands of Crete and Cyprus have suffered a recent bottleneck or colonization event. However, no bottleneck or founder effect was revealed by nuclear markers, thus indicating male-mediated gene flow from other populations that would increase nuclear genetic variability. Crete, and to a lower extent Cyprus, are thought to play a central role in such male-mediated gene flow that may reduce the negative effect of genetic drift or inbreeding on the small populations of Lebanon and Israel. This population structure indicates that assessing population relevance only on the basis of genetic variability and size would be misleading, as some populations not fulfilling those requirements may play a relevant role in genetic exchange and hence contribute to the overall genetic variability.

Keywords *Caretta caretta* · Mitochondrial DNA · Microsatellite DNA · Mediterranean · Genetic structure

Introduction

The circumtropical loggerhead sea turtle (*Caretta caretta*) is the most common sea turtle breeding in the Mediterranean sea (Broderick et al. 2002). It is estimated that about 5,000 nests are laid every year in the Mediterranean (Margaritoulis et al. 2003), much less than the large rookeries of the Atlantic (about 80,000

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nests/year; Ehrhart et al. 2003) or the Indian Ocean (about 20,000–40,000 females/year that represents about 70,000–140,000 nests/year; Baldwin et al. 2003). Most rookeries are located in the eastern Mediterranean basin (Margaritoulis et al. 2003) although sporadic nesting has been reported from the western basin (Llorente et al. 1992; Tomas et al. 2002; Delauguerre and Cesarini 2004).

The species is listed as ''endangered'' globally by the IUCN (IUCN 2006) although Groombridge (1990) considered the Mediterranean populations as ''critically endangered''. The Mediterranean population has declined due to incidental catch by fishing activity, egg harvest and tourism development (Margaritoulis et al. 2003). Moreover, genetic studies on Mediterranean loggerhead sea turtles (Bowen et al. 1993; Encalada et al. 1998) provided more arguments for considering the Mediterranean a relevant area for conservation of the loggerhead sea turtle as it is for green sea turtle, *Chelonia mydas* (IUCN 2006).

Population genetic studies with marine turtles first used the mitochondrial DNA (mtDNA) using restriction fragment length polymorphisms (RFLPs) (e.g. Bowen et al. 1992, 1993) and then focused on the control region of the mtDNA by sequencing (e.g. Bowen et al. 1998, 2005; Encalada et al. 1996, 1998; Laurent et al. 1998). The mtDNA is maternally inherited and high levels of genetic structuring were found all around the world either by RFLPs or by sequencing. These high levels of genetic structuring indicated that the females of marine turtles were highly philopatric to the beaches were they were born (Meylan et al 1990). Studies on the Mediterranean loggerhead sea turtle populations (Bowen et al. 1993; Encalada et al. 1998) indicated that these rookeries became isolated from the Atlantic populations at the beginning of the Holocene, and should be considered an independent management unit. Genetic isolation from the Atlantic populations is likely to further increase the vulnerability of the Mediterranean population, not only because the strength of stochastic phenomena increases, but also because of the potential reinforcement of inbreeding depression and loss of diversity through genetic drift. Furthermore, the existence of internal genetic structuring has been proposed to increase the probability of local extinctions in animal populations (Frankham et al. 2002). Although this has not been proved for marine turtles, the existence of several different management units within the Mediterranean, some of them of extremely small size (Margaritoulis et al. 2003; Khalil et al. in press), would make extinction risk even higher.

Nuclear markers (nDNA) were incorporated to marine studies first as RFLPs of anonymous nuclear loci (Karl et al. 1992), then as randomly amplified polymorphic DNA (RAPDs) (Schroth et al. 1996) and more recently as microsatellites (e.g. Bowen et al. 2005; FitzSimmons et al. 1997b), biparentally inherited markers that, combined with mtDNA, provide a further insight into the structure of marine turtle populations, as mtDNA ignores male-mediated gene flow. This combined approach has revealed that that the Atlantic populations of the loggerhead sea turtle in the northern hemisphere are highly structured with mtDNA, hence reflecting the high philopatry of the females, but no structure exists for the nDNA due to male-mediated gene flow. As a result, male-mediated gene flow would prevent genetic isolation in Atlantic loggerhead sea turtles despite female philopatry (Bowen et al. 2005). Similar results were reported by FitzSimmons et al. (1997b) for Australian green turtles.

Available information suggests that this may not be a likely scenario in the Mediterranean, as regional genetic structuring has been observed not only for mitochondrial DNA markers (Laurent et al. 1993, 1998; Schroth et al. 1996; Encalada et al. 1998), but also in nuclear DNA (Schroth et al. 1996). Unfortunately, sample effort has been uneven and some areas have been poorly sampled or not sampled at all. Without more detailed information on the genetic structure of the populations nesting in the eastern Mediterranean, identifying management units is difficult and may lead to inappropriate management decisions. The absence of internal structuring would indicate that the Mediterranean rookeries may be treated as a single management unit and hence, the loss of small local nesting sites would not hinder the conservation of the overall genetic variability. Otherwise, if internal structuring exists, several management units would be defined and should be preserved to guarantee healthy populations.

The purposes of this paper are (1) to assess the population structure of Mediterranean loggerhead populations, including gene flow and possible bottlenecks among the nesting sites, using both nuclear (nDNA) and mitochondrial (mtDNA) markers and (2) identify those populations that need to be protected in order to preserve the genetic diversity of the Mediterranean rookeries.

Methods

Collection and DNA extraction

During the nesting seasons of 2003 and 2004, hatchlings were sampled from 112 independent nests found in seven different nesting areas in the Mediterranean

(Fig. 1). Individuals were collected from Zakynthos, Lakonikos and Crete in Greece; Fethiye, in west Turkey; northern Cyprus; El Mansouri in Lebanon; and sites scattered along the Israeli coastline (Table 1). To avoid pseudoreplication (e.g. sampling hatchlings from two nests of the same female), sampled females were tagged with external flipper tags or subcutaneous PIT tags. When this was unfeasible, samples were collected only from clutches laid within a 15-day window, as females rarely nest at intervals shorter than this period. These procedures are expected to ensure independency of the collected samples. Furthermore, when dead embryo/hatchlings from a nest were found, only one was collected and analyzed. From each individual, muscle or skin samples were stored in 95% ethanol. DNA was extracted using the QIAamp extraction kit (QIAGEN®) following the manufacturer's instructions.

Mitochondrial DNA analysis

A fragment of 391 base-pair (bp) of the mtDNA control region was amplified by polymerase chain reaction (PCR) using the primer pairs TCR1 and TCR2 (Norman et al. 1994) as described in Carreras et al. (2006).

Sequences were aligned by eye using the program BioEdit version 5.0.9 (Hall 1999) and compared with haplotypes previously described (Bolten et al. 1998; Encalada et al. 1998; Carreras et al. 2006) and found in the Archie Carr Center for Sea Turtle Research database (http://accstr.ufl.edu/). Previous data from the Greek population of Kyparissia (Encalada et al. 1998) and from Cyprus and eastern Turkey (Laurent et al. 1998) were also included in the present work for statistical analysis (Table 1). In order to establish haplotype relationships, a haplotype network was constructed using package TCS v1.02 (Clement et al. 2000), which implements the statistical parsimony (SP) described in Templeton et al. (1992).

Genetic differentiation between different samples from the same location was assessed with the Chi-square test (Cuadras 1983) as implemented in the program CHIRXC (Zaykin and Pudovkin 1993). When statistical differences were not found between published and present data of the same nesting area, the two sets were considered to be subsamples of the same population. Both groups of studies were cautious in order to avoid pseudoreplication but, because independence of data across different sampling datasets cannot feasibly be totally ensured, the largest sample was considered for each nesting area, as detailed in Table 1.

Haplotype diversity (h), nucleotide diversity (π) and the genetic distance (γ_{st}) (Nei 1982) between each pair of populations were calculated using the program DNAsp (Rozas et al. 2003). Differentiation among population pairs within the Mediterranean was also assessed by the Chi-square test. A sequential Bonferroni correction was not applied for mtDNA nor for nDNA multiple pairwise comparisons, since they dramatically increase the probability for type II error (β: assume no differentiation when it does exist), an effect that becomes worse as many P-values are discarded (Perneger 1998; Cabin and Mitchell 2000; Moran 2003).

The number of migrants (N_m) between each population pair was calculated from genetic distances

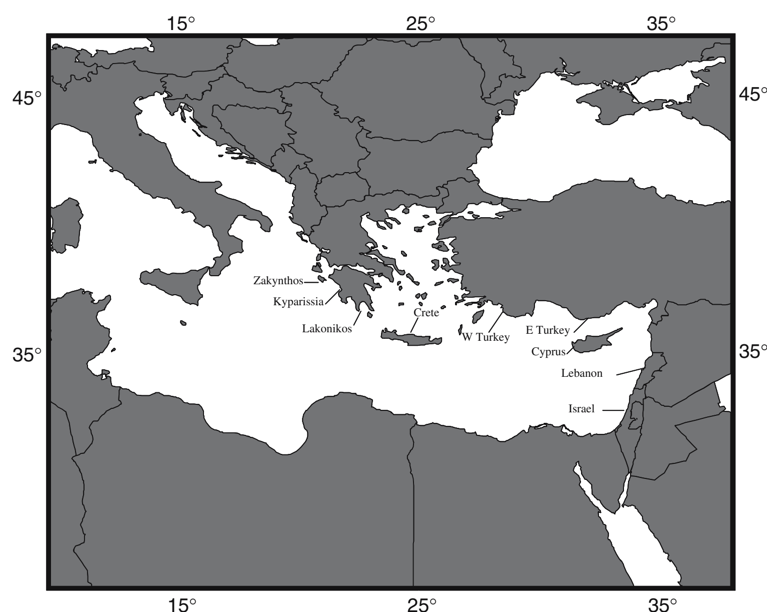

Fig. 1 Map of analyzed locations. See Table 1 for details of each location

Table 1 Sampling sites, number of individuals (n), percentage of mtDNA haplotypes and source

Sampling site	Acronyms	n	% of mtDNA haplotypes					Source
			CC-A2	CC-A3	CC-A6	CC-A29	CC-A32	
Zakynthos	ZAK	20	85	–	10	–	5	Present study
Kyparissia	KYP	21	90	–	10	–	–	Encalada et al. (1998)
Lakonikos	LAK	19	95	–	5	–	–	Present study
Crete	CRE	19	100	–	–	–	–	Present study
W Turkey	WTU	16	94	6	–	–	–	Present study
E Turkey	ETU	32	59	41	–	–	–	Laurent et al. (1998)
Cyprus	CYP	35	100	–	–	–	–	Encalada et al. (1998)
Cyprus	CYP	10	100	–	–	–	–	Present study
Lebanon	LEB	9	100	–	–	–	–	Present study
Israel	ISR	20	84	–	–	16	–	Present study

through the equation $Nm = \frac{1}{2}\left(\frac{1}{\text{Gamma}_{st}} - 1\right)$ (Takahata and Palumbi 1985).

Microsatellite analysis

Seven previously described microsatellite loci for sea turtles were used in this study: Cm84, Cc117, Cm72 and Ei8 (FitzSimmons et al. 1995); Cc141 and Cc7 (Bowen et al. 2005); and Ccar176 (Moore and Ball 2002). However, to improve amplification reaction and allele sizing the reverse primer of the locus Ccar176 was redesigned (Forward: 5′-GGCTGGGTGTCC-ATAAAAGA-3′ and new Reverse: 5′-CCCTAAG-TAAAGATTGGCTGCT-3′) using the sequence of the original clone found in GenBank (accession number AF333763). One primer for each pair was fluorescently labeled with NED, PET, VIC or 6-FAM. Each locus was amplified using a cycle of 95°C for 2 min 30 s followed by 30 cycles at 95°C for 45 s, 55°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 5 min. Allele length was determined on an ABI 3730 automated DNA Analyzer (Applied Biosystems). Allele sizes were assigned using the Genemapper package (ABI PRISM® GeneMapper™ Software ver. 3.0.).

We conducted pairwise tests for population differentiation (F_{st}), and departure from the Hardy–Weinberg equilibrium as well as for detecting linkage disequilibrium between loci. P-values for population differentiation were calculated with a Markov chain randomization (Guo and Thompson 1992). Fisher's method, which assumes statistical independence across loci, was used to combine test results for allelic counts among the populations for all seven loci (Raymond and Rousset 1995). A Mantel test was performed to detect isolation by distance between Mediterranean nesting areas. All these statistical analyses were conducted using Genepop ver. 3.4 (Raymond and Rousset 1995). Geographic distances for the Mantel test were calculated with the aid of the program ArcView ver. 3.1©. Furthermore, the program Bottleneck ver. 1.2 (Cornuet and Luikart 1996) was used to detect recent bottlenecks in populations using the two-phased model (TPM) as recommended by the authors for microsatellites consists of mostly one-step mutations, but a small percentage (5%–10%) of multi-step changes. Furthermore, genetic distances found in the Mediterranean nesting populations (present study) were compared to those found in the Atlantic nesting populations in the northern hemisphere, hence excluding Brasil (Bowen et al. 2005), using a T-test, as data met the normality and homoscedasticity requirements.

The number of migrants between each population pair (N_m) was calculated from the F_{st} values using the formula $Nm = \frac{1}{4}\left(\frac{1}{F_{st}} - 1\right)$ (Wright 1951). Estimates of gene flow based on F_{st} rely on the island model assumptions of equal population sizes and symmetric migration rates; however, these assumptions rarely hold in natural populations (Whitlock and McCauley 1999). Therefore we also estimated nuclear gene flow using the migration rate (M) that indicates how much more important migration is to bring new variants than mutation. We calculated this parameter using a maximum-likelihood method based on a coalescent approach (Beerli and Felsenstein 1999) implemented in MIGRATE ver. 1.7.6.1. (Beerli 2002). For all analyses, the default settings were used. Analyses were performed under the 'allele model' rather than the 'microsatellite model' because allele frequency distributions of these loci did not fit strict expectations of the SMM. Because convergence problems are common with Markov chain estimations, we performed each analysis five times, and the values presented herein are the mean of all replicate runs and the standard deviation across runs. Gene flow (N_m) obtained from mtDNA was compared with that obtained from nDNA using a Wilcoxon signed rank test.

Population structure was also assessed using the programme STRUCTURE ver. 2.1 (Pritchard et al. 2000), which implements a Bayesian clustering method to identify the most likely number of populations (K) without using prior information on sampling location. The program groups individuals in K populations so as to achieve Hardy–Weinberg and linkage equilibrium. The search strategy described in Evanno et al. (2005) was followed and 20 runs were carried out for each value of K (from 1 to 15). We set the length of the burn-in to 10,000 and MCMC (Markov Chain Monte Carlo) to 100,000 as preliminary tests showed that the results did not change substantially with longer values. We use the ad hoc statistic ΔK (Evanno et al. 2005) to detect the number of clusters in our sample but we also calculated Pr (X/K) as described in the manual.

Finally, we considered adult population size to test if this parameter can explain possible differences in genetic variability between populations. This parameter was estimated using mean annual nesting values found by Margaritoulis et al. (2003) and Khalil et al. (in press) and applying a correction factor of 1.48. This correction factor was calculated assuming equal sex ratio, a mean of 3.49 clutches per season and a mean of 2.59 years of remigration interval (Miller 1997).

Results

Mitochondrial DNA

A total of five haplotypes were found among the nesting areas investigated (Table 1). Haplotypes CC-A2 and CC-A3 were previously described and are shared by both the Mediterranean and Atlantic nesting beaches (Encalada et al. 1998; Laurent et al. 1998). Haplotype CC-A6 was also reported previously only in the Mediterranean nesting beaches (Encalada et al. 1998). Finally, haplotypes CC-A29 and CC-A32 had not been previously found in any nesting beach (GenBank accession numbers AY742910 and AY742913) but were recently reported from western Mediterranean feeding grounds (Carreras et al. 2006).

The haplotype network constructed using parsimony (Fig. 2) exhibited an ambiguity, as haplotype CC-A32 differed by one substitution either from haplotype CC-A3 (from eastern and western Turkey) or from CC-A6 (from Greece). The geographic location of the haplotypes allowed us to clarify this ambiguity since haplotype CC-A32 is only present in Zakynthos, a location that has haplotype CC-A6 but not haplotype CC-A3. Furthermore, CC-32 and CC-A6 share a gap and differ by a transition, while CC-32 and CC-A3 differ by the above-mentioned gap while sharing the transition. It is thus more probable that the transition has independently arisen twice.

Both the new and the previous data sets from Cyprus exhibited only haplotype CC-A2. As a consequence, since the earlier data set was much larger than our data set, we considered more convenient to use only the former in the analysis. They were not combined to fully avoid any risk of pseudoreplication. However, the two Turkish data sets were significantly different (Chi square, $P = 0.013$), although they shared the same haplotypes (CC-A2 and CC-A3). These sets came from nests collected from two different and distant areas within Turkey and, hence, they were considered independent and treated as different units in the analysis.

Although all populations shared haplotype CC-A2 (Table 1), pairwise genetic distances revealed highly significant differences between some of the nesting sites (overall $\gamma_{st} = 0.212$, $P < 0.001$, Table 2A for pairwise comparisons). The nesting sites at Zakynthos, Lakonikos and Kyparissia exhibited haplotypes CC-A2 and CC-A6 with highly similar percentages (Table 1) and differences found between them were not statistically significant (Table 2A). Consequently, these locations were all pooled as Greece (GRE) in subsequent analysis. Once the Greek populations were grouped, statistically significant differences were observed both with eastern Turkey and Israel (Table 2B). The nesting sites of Cyprus, Crete and Lebanon exhibited only haplotype CC-A2 (Table 1) and could be considered homogeneous, however these populations were not grouped as analysis with nuclear DNA (see next section) show differences between some of these populations. Genetic diversity was very different among nesting areas both in terms of haplotype diversity ($h = 0.000$–0.498) and nucleotide diversity ($\pi = 0.000$–0.00131) (Table 3). Estimates of gene flow for mtDNA were also highly variable and ranged from $N_m = 3.8$–55.1 (Table 4).

Microsatellites

All loci were highly polymorphic; allele numbers overall ranged from 6 (Cm72) to 15 (Cc7), with an average of 10.86 alleles per locus (Appendix Table 5). Some microsatellites (Cc141, Ccar176 and Ei8) failed to amplify in some specimens, probably due to the degraded state of several embryos. Thus, the sample size for these three markers in some populations was lower. Amplification problems were particularly severe for locus Ei8 as no sample amplified in Zakynthos

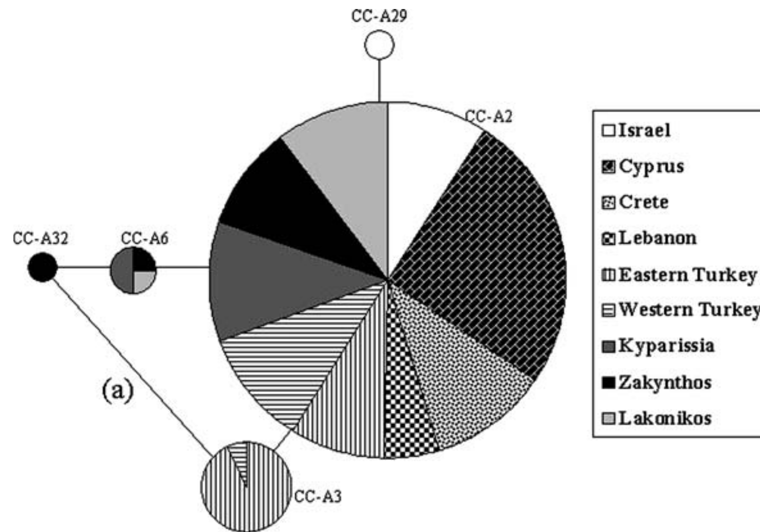

Fig. 2 Estimated parsimony network of mtDNA haplotypes. Each pie graph represents one haplotype and its frequency in each population. The size of each pie graph depends on its absolute frequency. Solid lines connect haplotypes by a single mutational change with a probability higher than 95%. Line (**a**) represents an ambiguity that could be resolved (see text for details)

Table 2 Genetic structure of the Mediterranean nesting populations

	ZAK	KYP	LAK	CRE	WTU	ETU	CYP	LEB	ISR
(A) Nesting populations before grouping									
ZAK		–	0.004	–0.003	0.022*	–	0.015*	0.014	0.022*
KYP	0.034		–	–	–	–	–	–	–
LAK	0.034	0.007		–0.002	0.004	–	–0.006	–0.002	0.015*
CRE	0.046	0.048	0.027		0.011*	–	0.004*	–0.004	0.007
WTU	0.022	0.041	0.030	0.035		–	0.005	0.008*	0.015**
ETU	0.096**	0.188**	0.188**	0.203**	0.127*			–	–
CYP	0.094*	0.067*	0.037	0.000	0.047	0.286***		0.004	0.013*
LEB	0.030	0.031	0.018	0.000	0.023	0.131*	0.000		0.010
ISR	0.061	0.073	0.070	0.086	0.067	0.181**	0.116**	0.057	
(B) Nesting populations after grouping ZAK, KYP and LAK as GRE									
	mtDNA GRE	nDNA GRE							
CRE	0.021	–0.005							
WTU	0.009	0.008*							
ETU	0.165***	–							
CYP	0.037	0.001**							
LEB	0.012	0.004*							
ISR	0.043**	0.016***							

Cells above the diagonal show genetic distances based on nDNA (F_{st} values), and those below the diagonal show genetic distances based on mtDNA (γ_{st} values). (–) no data is available. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

Table 3 Genetic variability in the Mediterranean loggerhead turtle nesting beaches

	mtDNA		nDNA			Nests per season[a]	*N*
	h	π	H_e	H_o	*k*		
GRE	0.19	6.6×10^{-4}	0.63	0.56	7.00	2,073 (1,250–3,184)	3,068 (1,850–4,712)
CRE	0	0	0.66	0.62	6.43	387 (315–516)	572 (466–764)
WTU	0.13	3.3×10^{-4}	0.65	0.68	5.71	124 (88–158)	183 (130–234)
ETU	0.50	1.31×10^{-3}	–	–	–	≅ 100	≅ 148
CYP	0	0	0.72	0.71	5.86	572 (404–775)	846 (598–1,147)
LEB	0	0	0.72	0.63	6.43	35 (33–37)	52 (49–55)
ISR	0.28	7.4×10^{-4}	0.70	0.67	7.00	33 (10–52)	49 (15–78)

Haplotype diversity (*h*), nucleotide diversity (π), gene diversity (H_e), observed heterozygosity (H_o) and mean allele number per locus (*k*). (*N*) Population size calculated from records of nests per season, as explained in the text. In brackets are shown the maximum and minimum registered values. Acronyms are defined in Table 1. (–) no data available

[a] Data from Margaritoulis et al. (2003) and Khalil et al. (in press)

Table 4 Estimates of gene flow between Mediterranean populations based either on mtDNA or nDNA and using different methods

	mtDNA	nDNA			
	γ_{st}	F_{st}	MIGRATE		
	N_m	N_m	M_{1-2}	M_{2-1}	M
GRE-CRE	23.3	–	18.7 (4.9)	7.7 (2.5)	26.4
GRE-WTU	55.1	31.0	15.9 (4.2)	8.8 (1.9)	24.8
GRE-CYP	12.7	249.7	14.3 (8.2)	4.4 (1.6)	18.7
GRE-LEB	41.2	62.2	2.4 (0.5)	2.2 (8.8)	4.6
GRE-ISR	11.1	15.4	22.2 (7.1)	12.8 (3.7)	35.0
CRE-WTU	13.8	22.5	21.1 (7.0)	15.6 (6.6)	36.7
CRE-CYP	–	62.2	9.8 (5.4)	6.5 (3.5)	16.2
CRE-LEB	–	–	1.5 (0.6)	7.5 (2.1)	9.1
CRE-ISR	5.3	35.5	10.2 (3.2)	11.4 (5.4)	21.6
WTU-CYP	10.1	10.1	10.6 (7.1)	8.8 (2.4)	19.4
WTU-LEB	21.2	31.0	3.8 (1.4)	9.0 (4.0)	12.8
WTU-ISR	7.0	16.4	10.2 (2.6)	6.1 (3.2)	16.3
CYP-LEB	–	–	1.5 (1.0)	5.5 (5.0)	7.0
CYP-ISR	3.8	19.0	6.5 (4.6)	8.4 (8.9)	14.9
ISR-LEB	8.3	24.7	2.0 (0.8)	9.8 (4.7)	11.8

(–)N_m was not calculated due to negative or null genetic distances. Results from MIGRATE are given as the migration rate from the first population to the second (M_{1-2}), from the second population to the first (M_{2-1}) and total migration rate (M). Standard Deviation across the five runs is given in brackets

(data not shown) and, for this reason, all the statistical tests were performed twice, with and without this locus. No differences were found in all the pair-wise comparisons involving Zakynthos with these two analyses. Hence, this locus was maintained as is informative for comparisons not involving Zakynthos.

No departure from Hardy–Weinberg equilibrium was detected for any population (Chi square, $P > 0.05$ in all cases). Furthermore, no linkage disequilibrium was found between loci pairs (Chi square, $P > 0.05$ in all cases) and hence, independence of loci was assumed. Observed heterozygosity was highly variable, ranging from $H_o = 0.03$ (cm72 in Greece) to $H_o = 0.94$ (cc7 in Israel) (Appendix Table 5). Gene diversity was also highly variable ranging from $H_e = 0.03$ (cm72 in Greece) to $H_e = 0.87$ (cc7 in Israel). However, mean values of H_e, H_o as well as mean number of alleles (k) were not different among populations (Friedman ANOVA, $P > 0.05$) despite the great differences in the reported size of the female populations (Table 3). Moreover, no recent bottleneck events were detected for any population under the TPM model (Wilcoxon test $P > 0.05$ in all cases).

Significant genetic structure was observed among all populations (overall $F_{st} = 0.005$, $P < 0.001$); but genetic differentiation between population pairs was only statistically significant for 9 out of 21 pair-wise comparisons (Table 2A). Cyprus and Crete exhibited significant differences based on microsatellites despite having the same mtDNA haplotype at 100% frequency (Table 2). The Greek populations were grouped since they were not genetically differentiated either with microsatellites or mtDNA. In addition the grouping did not depart from the Hardy–Weinberg equilibrium (Chi square, $P > 0.05$). Israel, Turkey, Greece and Cyprus were different from each other with the exception of Cyprus from Turkey. Lebanon was distinct from Greece and Turkey. Crete was different from Turkey and Cyprus but not from the rest (Table 2). Isolation by distance is an unlikely explanation for the genetic differences here observed, as no correlation exists between geographic location and genetic distance (Mantel test, $P = 0.080$). Genetic pairwise distances were significantly higher in the Mediterranean (present study) than those reported by Bowen et al. (2005) for the Atlantic (T-Student, $P < 0.001$).

Gene flow (N_m) between populations was highly variable (Table 4). Gene flow estimated from nDNA data was always significantly higher than that calculated from mtDNA (Wilcoxon signed rank test, $P < 0.001$). Gene flow calculated using the coalescent approach as implemented in MIGRATE (Table 4, Fig. 3) do not correlate with F_{st} estimates of gene flow (Mantel test, $P = 0.66$) as found in previous studies with marine turtles (Bowen et al. 2005). Hence, some population pairs that showed deep genetic differentiation using F_{st} methods, showed high levels of migration using the coalescent approach of MIGRATE (e.g. Greece and Israel).

Without using prior information on sampling location, the most probable number of populations estimated using STRUCTURE is 4 ($\Pr(X/K) = 0.95$ for $K = 4$). However, the ΔK ad hoc statistic (Evanno et al. 2005) suggested that the most probable number of populations is 2 with a secondary peak on $K = 5$. This

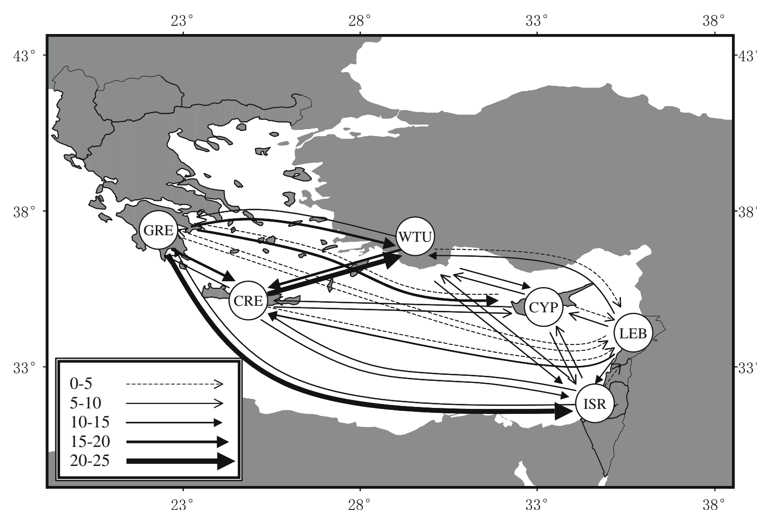

Fig. 3 Migration rate (M) between each pair of populations calculated using coalescent methods as implemented in MIGRATE. Each arrow width is proportional to MIGRATE values as detailed in Table 4

is the result expected for a Contact Zone model where populations (4 or 5) are partially connected (Evanno et al. 2005). When prior information of populations assuming the four units described by the F_{st} was used, all individuals of Greece, Cyprus, Israel and Turkey were reassigned to its origin population while samples of Crete and the Lebanon were not assigned to any of the former populations.

Discussion

Previous results on the genetic structure of two species of sea turtles have revealed a similar scenario: female philopatry generating a deep structuring for mitochondrial markers and male-mediated gene flow that assures wide dispersal of nuclear genes between populations (Bowen et al. 2005; FitzSimmons et al. 1997b). In other words, the loss a nesting beach would mean a loss of mitochondrial diversity and a loss of habitat, but nuclear diversity will be eroded only if a large number of nesting sites are lost (Bowen et al. 2005). The present study aimed to fill that void in knowledge about the loggerhead sea turtle in the Mediterranean and to improve its conservation there.

Population structure

The observed low variability of the mtDNA control region compared with that of the Atlantic populations (Bowen et al. 2005 and references therein) is probably due to the recent origin of the Mediterranean populations, which were founded by a few Atlantic migrants about 12,000 years ago (Bowen et al. 1993). Those colonizers are thought to carry with them only one or two of the several haplotypes found in the Atlantic populations. The haplotype CC-A2 was certainly one of them and was likely present from the beginning in all Mediterranean nesting beaches. The new haplotypes CC-A6 and CC-A29 evolved from it, but remained restricted to small regions within the eastern Mediterranean due to the philopatry of the females. The origin of haplotype CC-A3 is uncertain, as it may come from the Atlantic or may have evolved independently in the Mediterranean from haplotype CC-A2. Although there is no way to assess in the present study which is the right scenario, the transitional substitution that differentiates CC-A3 from CC-A2 took place at least twice: in the Atlantic it created haplotype CC-A3 from haplotype CC-A2 and in Greece it created haplotype CC-A32 from haplotype CC-A6, hence becoming the first example of homoplasy reported for the mtDNA control region in marine turtles. Thus, independent evolution of haplotype CC-A3 in the Atlantic and Turkey is a plausible hypothesis that might be addressed in the future using longer sequences. Whatever the origin of haplotype CC-A3, restricted gene flow, caused by the philopatry of females, limited the geographic expansion of haplotypes CC-A3, CC-A6, CC-A32 and CC-A29 within the Mediterranean.

Genetic differentiation based on mtDNA revealed the existence of at least four independent units within the eastern Mediterranean, most of them characterized by one exclusive haplotype: (1) the nesting beaches located on mainland Greece and the adjoining Ionian islands, characterized by haplotype CC-A6; (2) eastern Turkey, characterized by haplotype CC-A3; (3) Israel, characterized by haplotype CC-A29 and (4) Cyprus, comprising only the widespread CC-A2. The only

non-significant pairwise comparison between these units is that involving Cyprus and Greece although the *P*-value was very close to significance ($P = 0.06$) and non-grouped results showed differences between Cyprus and both Zakynthos and Kyparissia, the more distant Greek sampling areas. Probably, that difference will become statistically significant with a higher sample size and hence we consider this result as marginally significant. The existence of a single evolving unit including mainland Greece and the Ionian islands is also supported by tagging experiments demonstrating exchange of females between Greek nesting beaches (Margaritoulis 1998). Conversely, no evidence exists for female exchange between nesting beaches of the four above-reported units.

The data from the nDNA also support the status of the Greek, Israeli and Cyprian populations as independent units and indicates that the population from western Turkey is also a different unit, although closely related to that from Cyprus. Schroth et al. (1996) suggested, on the basis of nDNA (RAPDs) and mtDNA (control region) data, that Turkish rookeries differ from other Mediterranean rookeries and that an east-west cline exists along the Turkish coastline. Our results support such a hypothesis because differences for mtDNA have been found between eastern and western rookeries. Furthermore, western Turkey differs also for nDNA from most of the remaining Mediterranean nesting areas. However, as samples from eastern Turkey were not available for the present work, we cannot determine whether a cline exists also for microsatellite markers. Sampling in eastern Turkey would be advisable to clarify the status and the relationships of the loggerhead populations in the northeast shore of the Mediterranean. The genetic status of Crete among the rookeries is puzzling. The fact that Crete differs only from eastern Turkey with mtDNA but differs from Cyprus and western Turkey with nDNA may indicate that Crete is a stepping stone linking the nesting populations in the latitudinal extremes of the eastern Mediterranean, i.e. Greece and Israel. Small sample size impedes resolving the relations of the Lebanese population, but it probably forms a unit with the nearby Israeli coast, as (1) no difference exists either for mtDNA or nDNA, (2) both differ from the remaining populations and (3) the El-Mansouri beach, in Lebanon, is close to the Israeli border. The absence of the haplotype CC-A29 from the Lebanese population is likely an artifact due to small sample size.

The small size of some populations (Margaritoulis et al. 2003; Khalil et al. in press) is the main problem when sampling nesting sites of the loggerhead sea turtles in the Mediterranean, as it is not feasible to obtain the desired number of samples without jeopardizing sample independence. A further difficulty is poor sample quality, because tissues from nesting beaches are typically collected from dead embryos once live hatchlings had emerged. This sampling procedure is less invasive, but specimens are sometimes partially degraded and the genomic extraction or the DNA amplification for some loci is occasionally unsuccessful. In this study, problems in amplification were severe at Zakynthos for locus Ei8, as emergence events on this island are more scattered through time and, in order to maximize nest success, we excavated nests later than in other areas. For the green turtles (*Chelonia mydas*) in the Atlantic and the Pacific, Roberts et al. (2004) suggested that low sample size would lead to a lack of statistical power in detecting differentiation. Hence, we can be sure that the significant differences observed with low sample size are real, but the existence of non-significant values should not be considered to prove a lack of isolation because those results might be an artifact due to low sample size. For all these reasons, results for populations with low sample size (as Lebanon for both markers, or Cyprus for nDNA) should be taken with caution.

Regardless of those shortcomings and unresolved questions, the F_{st} values are an order of magnitude higher than those found between the North Atlantic nesting areas, indicating that the level of nDNA differentiation in the eastern Mediterranean is much higher than that previously reported for the North Atlantic nesting areas (Bowen et al. 2005).

Implications for conservation

The population structuring in the eastern Mediterranean has strong implications for the management of loggerhead sea turtles in the region. At a first glance, mtDNA data (Bowen 2003 and the present study) as well as the information on the number of nesting females (Margaritoulis et al. 2003) might indicate the existence of two groups of populations that might be subject to genetic depression: (1) the extremely small populations from Lebanon and Israel (Margaritoulis et al. 2003; Khalil et al. in press) and (2) the populations from Crete and Cyprus, where only the ancestral CC-A2 haplotype has been found (Laurent et al. 1998; Kaska 2000; present study). Populations with similar low levels of mtDNA diversity have also been observed in the Atlantic population of North Carolina, where only haplotype CC-A1 was detected (Encalada et al. 1998). Bowen (2003) hypothesized that this was due the recent northward expansion of the nesting effort along the North-American coastline, which was in turn

caused by the increase in temperature that occurred throughout the Holocene. However, a different hypothesis is needed for justifying the low diversity of the populations from Cyprus and Crete, as the latitude of the nesting beaches in both islands is lower than that of the more genetically diverse nesting beaches in Greece and Turkey. Interestingly, the coastlines of both Cyprus and Crete are rough and steep and the continental shelf around them is extremely narrow and is not connected to the shelf of the mainland (Hofrichter et al. 2004). As a consequence, the availability of suitable nesting beaches in these islands may have been dramatically affected by the sea level fluctuations that occurred throughout the Holocene (Tichy et al. 2004) and that likely caused a number of local extinctions which may have potentially lead to the low mtDNA genetic diversity observed. However, that pattern cannot be generalized to those islands surrounded by a wide continental shelf linking them to the mainland, like Zakynthos, in Greece. That island has one of the highest mtDNA genetic diversities recorded in the Mediterranean, probably because the fluctuations of the sea level are less likely to affect the availability of the nesting sites there due to the width of the continental shelf and the connection with the mainland.

Remarkably, all the above reported populations exhibit a high nuclear variability, either in terms of observed heterozygosity, gene diversity and allele number, which is statistically similar to that observed in the larger or more mtDNA diverse populations from Greece and Turkey. Moreover, nDNA did not reveal any recent bottleneck, either for the populations of Israel and Lebanon or for those of Cyprus and Crete. That result is striking considering that the populations in the Levantine shore of the Mediterranean, Israel and Lebanon, suffered a recent and huge reduction of their size due to intense harvest from World War I to mid 30s (Sella 1982). This paradox may have two non-exclusive explanations. The first one invokes that population decline in Israel and Lebanon is too recent to allow us to detect changes in genetic diversity, as only a few generations have passed for the long living turtles. This is an unlikely explanation, as allele numbers are expected to decline faster than heterozygosity after a bottleneck (Nei et al. 1975), but no differences in allele numbers or as heterozygosity was found between the populations that were harvested (Israel and Lebanon) and those that were not (Greece, Turkey or Crete). The alternative hypothesis is a male-mediated gene exchange with Crete (both Israel and Lebanon) and Cyprus (Lebanon only). Such a hypothesis is supported by the moderate migration rate revealed by MIGRATE and the indication by STRUCTURE that the eastern Mediterranean is a Contact Zone formed by several populations partially connected. In this scenario, Crete would play a central role, as it ensures gene flow between the large Ionian populations (Greece) and the tiny Levantine ones (Israel and Lebanon). Conversely, the population from Cyprus would link the Lebanese and the Turkish populations. Such a gene flow may be sex biased as nDNA gene flow is significantly higher than mtDNA gene flow. This is not unprecedented, as in every case published to date genetic structuring of sea turtles is deeper with mtDNA than with nDNA (FitzSimmons et al. 1996; Schroth et al. 1996; Bowen et al. 2005). This pattern can be partially explained by the haploid inheritance of mtDNA, imparting a four-fold lower effective population size relative to diploid nuclear loci (Birky et al. 1983). However, other factors must be invoked when values of population differentiation differ by an order of magnitude or more (Roberts et al. 2004; Bowen et al. 2005) as in this case. All these evidences indicate the existence of male-mediated gene flow between different mtDNA-defined populations on that the philopatry of males is less stringent than that of females. Similar scenarios have been reported for the same species in the western Atlantic Ocean (Bowen et al. 2005) and for the green sea turtle (*Chelonia mydas*) in Australia (FitzSimmons et al. 1996, 1997a, b).

Pulliam (1988) and Pulliam and Danielson (1991) first proposed the idea that populations with a positive growth rate (known as sources) may supply with recruits adjoining populations with a negative growth rate (sinks), thus generating a gene flow from the former to the latter. Shanker et al. (2004), on the basis of mtDNA, suggested a population structure for the olive ridley marine turtle (*Lepidochelys olivacea*) including source populations in the Indian Ocean and sink populations in the Pacific and Atlantic Oceans. These results here reported for the loggerhead sea turtles in the Mediterranean suggest that Greece acts as a source of nuclear genes for other populations through Crete, perhaps because of the size of the Greek population and stability of the beaches in evolutionary time. However, female exchange between nesting units seems unlikely on the basis of the available mtDNA data, hence indicating that nuclear genes, but not individuals, move from Greece to the remaining populations.

These conclusions highlight the relevance of detailed genetic analysis to ensure proper management of threatened species, as biases may have negative consequences. The application of genetic data to biodiversity conservation is often coarse, and populations without private alleles or haplotypes are thought to

deserve lower attention (Ruckelshaus et al. 2003). Indeed, if only mtDNA data were available, conservation efforts would focus on the nesting beaches that support populations with exclusive haplotypes, i.e. Greece, Turkey and Israel. On the other hand, if only nDNA data were available, the conservation of the rookeries in Greece, Israel, Turkey and, perhaps, Cyprus, would be considered enough to preserve the genetic variability of the loggerhead sea turtle in the Mediterranean as a whole. None of these alternatives would focus on the conservation of the nesting sites in Crete because they contain only a widespread mtDNA haplotype and share almost all nDNA alleles with other sites (Appendix Table 5). This would be a mistake, because this island, as well as Cyprus, might link the Levantine and Ionian populations by means of male-mediated gene flow, a conclusion that emerges only when multiple genetic approaches are combined.

For all those reasons, the genetic variability of the loggerhead sea turtle in the eastern Mediterranean will be preserved only if (1) all the mitochondrial variability is preserved, (2) all the nuclear variability is preserved and (3) the stepping stones connecting populations are preserved. We can thus conclude that (1) Greece, Turkey, and Israel should be preserved as they are different units in terms of mtDNA, (2) the same populations as well as Cyprus are not expendable because they are units in terms of nDNA, and (3) Crete and Cyprus should also be a priority because they link at least two of the former units. The relevance of the Lebanese population is a non-resolved issue due to small sample size but probably it forms a panmictic unit with Israel. Clearly, further research is needed to clarify the status of that population.

As a final remark we can extract that different markers should be used in order to get a deep knowledge of population structure and hence assess which ones form different units. Furthermore, not only genetically different populations deserve protection but also those allowing gene flow between them. In this way, negative effects of population reduction due to stochastic phenomena will be minimized and the genetic variability of the species will be preserved.

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Appendix

Table 5 Relative frequencies of alleles, number of chromosomes (N), number of alleles (k), gene diversity (H_e) and observed heterozygosity (H_o) in each population and for each locus

	Allele	GRE	CRE	WTU	CYP	ISR	LEB	Mean
cc141								
N		56	30	28	18	24	16	
k		7	6	6	6	9	9	7.167
	179	0	0	0	0	0	0.063	
	187	0.054	0.1	0.143	0	0.042	0	
	195	0	0	0	0	0.042	0.063	
	197	0.375	0.333	0.429	0.333	0.208	0.438	
	199	0.089	0.1	0.179	0.167	0.292	0.063	
	201	0.179	0.267	0.036	0.111	0.125	0.125	
	203	0.196	0.167	0.179	0.222	0.125	0.063	
	205	0.089	0.033	0	0.056	0.083	0.063	
	207	0.018	0	0	0	0.042	0	
	211	0	0	0	0	0	0.063	
	213	0	0	0.036	0.111	0.042	0.063	
H_o		0.643	0.6	0.857	0.778	0.917	0.625	0.737
H_e		0.784	0.795	0.757	0.83	0.862	0.817	0.807
cm72								
N		74	36	30	20	38	16	
k		2	2	2	3	3	3	2.5

Table 5 continued

	Allele	GRE	CRE	WTU	CYP	ISR	LEB	Mean
	223	0.986	0.972	0.9	0.8	0.947	0.813	
	233	0	0	0.1	0	0	0	
	241	0	0.028	0	0.15	0.026	0.125	
	243	0.014	0	0	0	0	0	
	245	0	0	0	0.05	0	0	
	247	0	0	0	0	0.026	0.063	
H_o		0.027	0.056	0.2	0.4	0.105	0.375	0.194
H_e		0.027	0.056	0.186	0.353	0.104	0.342	0.178
cm84								
N		68	36	28	20	34	18	
k		6	6	5	5	7	8	6.167
	311	0	0	0	0	0	0.056	
	313	0.529	0.500	0.357	0.5	0.294	0.222	
	315	0.191	0.250	0.429	0.25	0.235	0.333	
	317	0.029	0	0.036	0	0.088	0.056	
	319	0.015	0	0	0	0	0.056	
	321	0.118	0.194	0.071	0.1	0.147	0.056	
	323	0.118	0.056	0.107	0.1	0.176	0.167	
	325	0	0.028	0	0.05	0.029	0.056	
	327	0	0	0	0	0.029	0	
H_o		0.618	0.556	0.857	0.7	0.765	0.667	0.694
H_e		0.664	0.727	0.696	0.7	0.82	0.843	0.742
cc7								
N		78	36	32	20	32	18	
k		12	8	7	9	11	8	9.167
	167	0.103	0.278	0	0.1	0.063	0.056	
	171	0.103	0.111	0.188	0.1	0.281	0.056	
	173	0.231	0.139	0.25	0.35	0.125	0.222	
	175	0	0	0.031	0	0	0	
	177	0.026	0	0.063	0	0.031	0	
	179	0.013	0	0	0	0	0	
	183	0.192	0.056	0.25	0.1	0.063	0.056	
	185	0.026	0	0	0.1	0	0	
	187	0.064	0.083	0	0.05	0.188	0.056	
	189	0	0.028	0	0	0.031	0.111	
	191	0.013	0	0	0.05	0.031	0	
	193	0.013	0.028	0.031	0	0.125	0.056	
	197	0.205	0.278	0.188	0.1	0.031	0.389	
	199	0	0	0	0.05	0.031	0	
	201	0.013	0	0	0	0	0	
H_o		0.821	0.722	0.75	0.9	0.938	0.778	0.818
H_e		0.851	0.825	0.825	0.863	0.869	0.817	0.842
ccar176								
N		56	32	28	20	32	18	
k		8	8	4	7	6	6	6.5
	168	0	0	0	0.05	0	0	
	170	0	0	0	0.05	0.031	0	
	172	0	0	0	0	0.031	0	
	176	0.143	0.188	0.179	0	0.25	0.111	
	178	0	0.063	0	0.05	0.063	0.056	
	184	0.036	0.031	0.179	0.3	0	0	
	186	0.625	0.563	0.607	0.45	0.531	0.667	
	190	0.018	0.031	0	0	0	0	
	192	0.018	0	0	0	0	0.056	
	196	0.054	0.031	0.036	0.05	0.094	0.056	
	198	0.089	0	0	0	0	0.056	
	200	0	0	0	0.05	0	0	
	206	0	0.031	0	0	0	0	
	212	0.018	0.063	0	0	0	0	
H_o		0.5	0.813	0.643	0.7	0.688	0.556	0.65

Table 5 continued

	Allele	GRE	CRE	WTU	CYP	ISR	LEB	Mean
H_e		0.586	0.657	0.587	0.732	0.661	0.562	0.631
cc117								
N		76	36	26	20	38	18	
k		7	8	8	5	8	5	6.833
	231	0.039	0.111	0.077	0	0	0	
	234	0	0	0	0	0.026	0	
	235	0.487	0.444	0.5	0.5	0.395	0.333	
	237	0.053	0.083	0.077	0.05	0.105	0.056	
	239	0.092	0.028	0.038	0.05	0.026	0.111	
	241	0.171	0.111	0.077	0.15	0.211	0.278	
	243	0	0.028	0.038	0	0	0	
	246	0	0	0.038	0	0.026	0	
	247	0.145	0.139	0.154	0.25	0.184	0.222	
	253	0.013	0.056	0	0	0.026	0	
H_o		0.605	0.778	0.615	0.6	0.789	0.667	0.676
H_e		0.709	0.768	0.732	0.695	0.772	0.791	0.745
Ei8								
N		30	30	28	14	24	16	
k		7	7	8	6	5	6	6.5
	178	0	0.067	0.107	0.071	0.167	0.063	
	180	0.2	0.233	0.107	0.286	0.125	0.313	
	184	0.133	0.067	0.071	0	0	0	
	186	0.033	0.1	0.071	0.143	0	0.125	
	188	0.267	0.267	0.393	0.143	0.292	0.188	
	190	0.067	0	0.036	0	0	0.125	
	192	0.3	0.267	0.107	0.286	0.333	0.188	
	194	0	0	0.107	0.071	0.083	0	
H_o		0.733	0.8	0.857	0.857	0.5	0.75	0.75
H_e		0.818	0.825	0.817	0.846	0.786	0.85	0.824

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