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Phylogeography and population structure of the Atlantic and Mediterranean green turtle *Chelonia mydas*: a mitochondrial DNA control region sequence assessment

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Abstract

Mitochondrial (mt) DNA sequences were analysed to resolve the phylogeography and population genetic structure of Atlantic and Mediterranean populations of green turtles (*Chelonia mydas*). Analysis of sequence variation over 487 base pairs of the control (D-loop) region identified 18 haplotypes among 147 individuals from nine nesting populations. Pairwise comparisons of haplotype frequencies distinguished most nesting colonies, indicating significant genetic differentiation among rookeries and a strong propensity for natal homing behaviour by nesting females. Comparison of control region sequence data to earlier restriction fragment length polymorphism (RFLP) data for the same individuals demonstrates approximately a sixfold higher substitution rate in the 5′ end of the control region. The sequence data provide higher resolution both in terms of the number of mtDNA genotype variants and the phylogeographic relationships detected within the Atlantic region, and reveal a gene genealogy that distinguishes two groups of haplotypes corresponding to (i) the western Caribbean and Mediterranean, and (ii) eastern Caribbean, South Atlantic and West Africa. The data suggest that phylogeographic patterns in the Atlantic Ocean may be interpreted in terms of female nest site fidelity and episodic dispersal events. The distribution of mtDNA haplotypes within the region is thus explained by the geological and climatic alternations (glacial and interglacial) over the last million years.

Keywords: *Chelonia mydas*, control region, dispersal biogeography, mitochondrial DNA, phylogeography, sequencing, PCR

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Introduction

Mitochondrial (mt) DNA genealogies have been applied extensively to the study of maternal population structure and in many cases have revealed genetic partitions that are readily interpreted in terms of habitat distribution and geography (Avise 1994 and references therein). These population architectures appear to be influenced by many factors intrinsic to the life histories of the particular organisms, such as the mating system and the gene flow regime, but are also influenced by historical biogeographic and demographic factors. The study of intraspecific gene genealogies (i.e. phylogeography; Avise *et al.* 1987) allows us to test alternate hypotheses concerning the relationships of biota in separate geographical regions, and assess the relative impact of historical vicariant events and/or dispersal events on the contemporary distribution of evolutionary lineages.

The herbivorous green turtle (*Chelonia mydas*) is distributed circumglobally in tropical and subtropical oceans

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(Carr 1967). Exchange between separate ocean basins is limited by continental barriers but possible in principle around the Cape of Good Hope, a known passage route for tropical species (Briggs 1974). Within each ocean basin, mature females travel from feeding grounds to nesting beaches in reproductive migrations that span hundreds or thousands of kilometres (Mortimer & Carr 1987; Limpus *et al.* 1992). Despite this high dispersal potential and broad geographical distribution, tagging studies show strong segregation of females among the different nesting beaches (Carr *et al.* 1978; Balazs 1980; Limpus *et al.* 1992). These discrete populations are not separated by obvious geographical or climatic barriers, but instead appear to be shaped by the female's strong nest site fidelity, a process by which females return faithfully to the same rookery to nest in consecutive breeding seasons (Carr 1967). How might these behavioural aspects of sea turtle life history, as well as other demographic factors influence the current geographical distribution of green turtle lineages?

An earlier survey of green turtle population structure with mtDNA restriction fragment length polymorphisms (RFLPs) revealed a fundamental bifurcation of mtDNA lineages between Atlantic–Mediterranean and Indo-Pacific nesting colonies and a marked geographical structuring of mtDNA genotypes among Atlantic rookeries (Bowen *et al.* 1992). Despite the relatively high degree of resolution that mtDNA RFLP analyses have provided for other intraspecific surveys (Avise *et al.* 1987; Moritz *et al.* 1987; Harris 1989; Avise 1994), this approach yielded only eight haplotypes in Atlantic green turtles, too few to resolve intraoceanic phylogeographic patterns and certain aspects of population structure.

Here we employ mtDNA control region sequences to assess the population genetic structure and phylogeography of green turtles in the Atlantic Ocean and Mediterranean Sea. This study extends earlier descriptions of mtDNA control region sequence variation on a more limited (Caribbean) geographical range (Allard *et al.* 1994; Lahanas *et al.* 1994). mtDNA sequences are characterized by a rapid rate of evolution and lack of recombination (Brown 1983), features which are useful for the study of local population differentiation. In addition, the maternal mode of inheritance renders mtDNA appropriate for the study of genetic differentiation in C. *mydas*, a species with possible gender-biased dispersal (see Karl *et al.* 1992). These data also allow us to compare the level of genetic variation and haplotype relationships between the RFLPs examined by Bowen *et al.* (1992) and the control region sequences, and to propose a phylogeographic scenario that accounts for the current geographical distribution and historical patterns of dispersal of Atlantic green turtle mtDNA lineages. The inferences drawn from such historical, phylogenetic perspectives contribute toward a greater understanding of the influence of historical demographies on contemporary genome structures (Avise 1995).

Materials and methods

Mitochondrial DNA sequences were analysed for eight Atlantic and one Mediterranean populations of green turtles (Fig. 1). The mtDNA samples from Brazil, Ascension Island, Guinea Bissau and Cyprus were the same as those used by Bowen *et al.* (1992) and Karl *et al.* (1992) in earlier analyses of mtDNA RFLPs and nuclear DNA, respectively. Samples from Mexico were collected from X'Cacel and adjacent Isla Cozumel (Quintana Roo) during the 1993

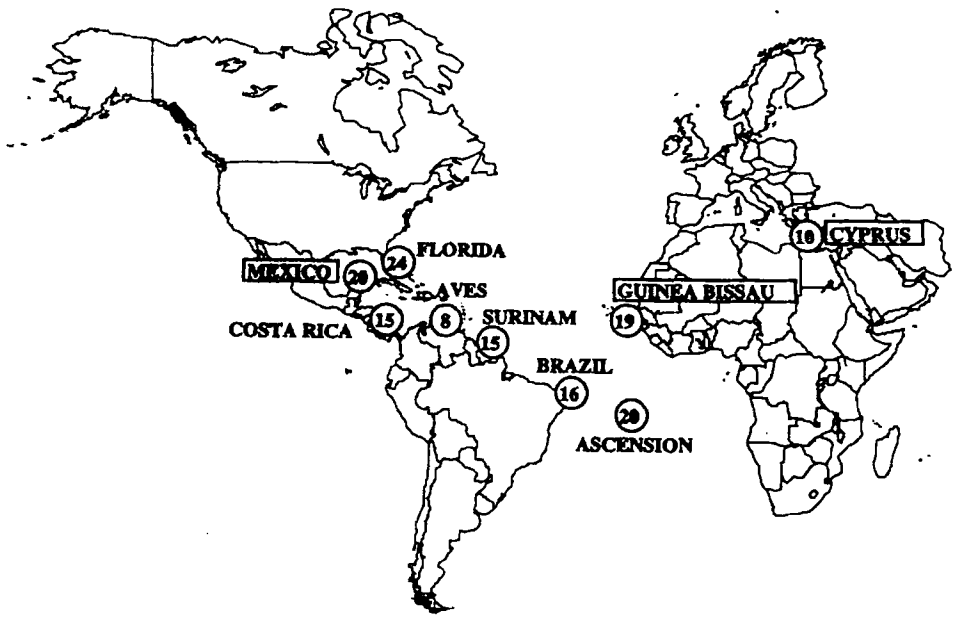

Fig. 1 Sample locations for *Chelonia mydas* (refer to text for nesting beach locales). Numbers refer to sample sizes for each locale.

nesting season, as blood from nesting females or hatchlings, obtained from the dorsal cervical sinus region (Owens & Ruiz 1980). The sequence orthologs from these five populations are combined here with those of other four previously analysed colonies: Florida, Costa Rica, Aves Island and Surinam (Allard *et al.* 1994; Lahanas *et al.* 1994), for a total of 147 individuals. Sample sizes for each collection locale are given in Fig. 1. A single individual from Queensland, Australia was included as an outgroup for phylogenetic analyses (sample also from Bowen *et al.* 1992).

Laboratory procedures

Samples from Brazil, Ascension Is., Guinea Bissau and Cyprus (for Mexico see below) were amplified by the polymerase chain reaction (PCR; Mullis & Faloona 1987) using primers LTCM2 (5'-CGG TCC CCA AAA CCG GAA TCC TAT-3') and HDCM2 (5'-GCA AGT AAA ACT ACC GTA TGC CAG GTT A-3'). These primers are longer versions of primers LTCM1 and HDCM1 developed by Allard *et al.* (1994) specifically for green turtles, and were designed to target an area of 510 bp of the 5' end of the control region. Amplified double-stranded mtDNA from the four populations was purified with Amicon centricon centrifugal microconcentrators (Centricon-100) and sequenced directly using the dideoxynucleotide chain-termination method (Sanger *et al.* 1977), following protocols provided with the ds-DNA Cycle Sequencing System of Bethesda Research Laboratories. Each template was sequenced with ^{32}P-labelled primers LDCM1 and LTCM2 (refer to Allard *et al.* 1994 for annealing positions). Fragments were separated in polyacrylamide gels (6%), air-dried and visualized by autoradiography.

The blood samples from the Mexican localities of X'Cacel and Isla Cozumel were collected following a protocol modified from White & Densmore (1992) and preserved in lysis buffer (100 mM Tris-HCl, pH 8; 100 mM EDTA, pH 8; 10 mM NaCl; 1.0% SDS) prior to processing. Mitochondrial DNA was isolated from the blood samples by a series of phenol/chloroform extractions (protocol modified from Hillis *et al.* 1990) and resuspended in 1 × TE buffer. Biotinylated versions of primers LTCM2 and HDCM2 were used to amplify the same 510-bp 5' end fragment of the mtDNA control region using PCR methodology (Innis *et al.* 1990). These primers contained a short sequence extension with complementarity to universal M13 forward (or reverse) primers, to facilitate sequencing using a dye-primer sequencing procedure (see below). Double-stranded PCR products were purified with Dynal streptavidin coated magnetic particles (Promega) for removal of excess reagents and denatured with 0.2 N NaOH. Single-stranded sequencing reactions were conducted with fluorescently labelled M13 primers in a robotic

Table 1 Polymorphic sites corresponding to 18 Atlantic and Mediterranean green turtle haplotypes. These polymorphisms include 17 transitions, two transversions, and one 10-bp repeat. Transversions are found in sites 355 and 436, the latter site also including a transition. A single individual from Cyprus was heteroplasmic at site 167

	Base positions																		
Haplotype	164	167	204	220	242	243	255	321	343	355	365	436	438	442	481	502	504	Repeat	558
I	C	A	G	G	G	A	T	T	C	T	C	C	G	G	T	A	G	-	A
II	C	A	G	G	G	G	T	T	C	T	C	C	G	G	T	A	G	-	A
III	T	A	G	G	G	A	T	T	C	T	C	C	G	G	T	A	G	-	A
IV	T	A	G	G	G	A	T	T	C	T	C	T	G	G	T	A	G	-	A
V	C	G	A	G	G	A	T	C	C	G	C	C	A	G	C	G	A	-	G
VI	C	G	A	G	G	A	T	C	C	G	C	C	A	G	C	G	A	-	A
VII	C	G	A	G	G	A	T	C	C	G	C	C	A	G	C	G	A	+	G
VIII	C	G	G	G	G	A	T	C	C	G	C	C	A	G	C	G	A	-	A
IX	C	G	G	G	G	A	T	C	C	G	C	C	A	G	T	G	A	-	A
X	C	G	G	G	G	A	T	C	C	G	T	C	A	G	C	G	A	-	A
XI	C	G	G	G	A	A	T	C	C	G	C	C	A	G	C	G	A	-	A
XII	C	G	G	G	G	A	T	C	T	G	C	C	A	G	C	G	A	-	A
XIII	C	A	G	A	G	A	T	C	C	T	C	C	G	G	T	A	G	-	A
XIV	C	A	G	A	G	A	T	C	C	T	C	A	G	G	T	A	G	-	A
XV	T	A	G	G	G	A	C	T	C	T	C	C	G	G	T	A	G	-	A
XVI	C	A	G	A	G	A	T	C	T	T	C	C	G	G	T	A	G	-	A
XVII	C	A	G	A	G	A	T	C	C	T	C	C	G	A	T	A	G	-	A
XVIII	C	G	G	G	G	A	T	T	C	T	C	C	G	G	T	A	G	-	A
Heteroplasmy	C	A/G	G	A	G	A	T	C	C	T	C	C	G	G	T	A	G	-	A

work station (Applied Biosystems model 800). The strand complementary to the biotinylated strand was sequenced. The labelled extension products were analysed with an automated sequencer (Applied Biosystems model 373 A) in the DNA Sequencing Core at the University of Florida. Sequencing runs were performed with forward and reverse primers for those samples for which presumptive polymorphisms needed to be confirmed (Ferl *et al.* 1991). Data were scored directly from the chromatogram output.

For both direct and automated sequencing, standard precautions, including the use of negative controls (template-free reactions) were used in the PCR amplifications to guard against contamination. Laboratory procedures for samples from the other four populations are described elsewhere (Allard *et al.* 1994; Lahanas *et al.* 1994).

Data analysis

Due to the extensive similarities of the individual sequences, these were aligned by eye. Estimates of within population genetic variation were obtained for each of the nine colonies in the form of haplotype ($\hat{h}$) and nucleotide ($\hat{\pi}$) diversities (Nei 1987: eqns 8.4 and 10.5, respectively). A chi-square test of independence (Sokal & Rohlf 1981) using the Monte Carlo randomization method (Roff & Bentzen 1989) in the program CHIRXC (Zaykin & Pudovkin 1993), was used to test for significant differences in haplotype frequencies between pairs of populations. The sequential Bonferroni procedure suggested by Rice (1989) was used to correct for multiple pairwise comparisons.

Estimates of nucleotide sequence divergence (p-values) between mtDNA genotypes were calculated with the Kimura two-parameter method (Kimura 1980), and the resulting distances were clustered using the neighbour-joining (NJ) (Saitou & Nei 1987) and UPGMA algorithms (Sneath & Sokal 1973) provided by MEGA (Kumar *et al.* 1993). The simplicity of this data set also allowed the haplotypes to be linked by eye into an unrooted parsimony network. Maximum parsimony analysis using heuristic searches and bootstrapping were also performed with PAUP (Swofford 1993).

Migration estimates between pairs of nesting colonies were calculated from *Gst* values (Nei 1987: p. 191) using the equation $Nm = 0.5(1/Gst-1)\,(L-1/L)^2$, where L is the number of populations, as defined by Takahata & Palumbi (1985). Average migration rates across all populations were calculated by the private allele method (Slatkin 1985; Barton & Slatkin 1986) using the equation in Slatkin & Barton (1989).

Results

The sequences were aligned for 487 bases from the 5′ end of the control region. A total of 20 polymorphisms were found at 19 polymorphic sites, corresponding to 17 transitions, two transversions, and one 10-bp repeat (Table 1). Site 436 showed two kinds of base substitutions including a transition (C→T) and a transversion (C→A), based on the predominance of cytosine at this position in all other haplotypes. Nucleotide substitutions throughout the 487-bp sequence showed a transition:transversion ratio of

Table 2 Distribution of green turtle haplotypes in the Atlantic Ocean and Mediterranean Sea. The Roman numerals represent mtDNA haplotypes defined in Table 1. Asterisk denotes an individual heteroplasmic for an additional site change at site 167

Haplotype	Florida	Mexico	Costa Rica	Aves	Surinam	Brazil	Ascension	Guinea Bissau	Cyprus
I	11	7							
II	1								
III	12	5	14	1					
IV			1						
V		1		7	13				
VI					1				
VII					1				
VIII						8	16	19	
IX						5	1		
X							3		
XI						1			
XII						2			
XIII									9*
XIV									1
XV		1							
XVI		1							
XVII		2							
XVIII		3							

Table 3 Haplotype ($\hat{h}$) and nucleotide ($\hat{\pi}$) diversities for nine Atlantic populations of green turtles

Population	Haplotype diversity ($\hat{h}$) ± standard error	Nucleotide diversity ($\hat{\pi}$)
Florida	0.56 ± 0.047	0.0013
Mexico	0.82 ± 0.058	0.0057
Costa Rica	0.13 ± 0.11	0.00028
Aves	0.25 ± 0.18	0.0053
Surinam	0.26 ± 0.14	0.00056
Brazil	0.68 ± 0.085	0.0017
Ascension Island	0.35 ± 0.12	0.00077
Guinea Bissau	0.00	0.00
Cyprus	0.22 ± 0.16	0.00042
Overall	0.83	0.0050

8.5 : 1, in accordance with the range of substitution biases reported for other mtDNA sequences comparisons (Brown *et al.* 1982). The 10-bp repeat found in one specimen from Surinam is treated throughout our analysis as a single polymorphism. Based on these control region polymorphisms, 18 distinct haplotypes were recognized among the 147 individuals from nine assayed populations (Table 2). A single individual from Cyprus was identical to haplotype XIII except for apparent heteroplasmy at site 167. This individual was treated as haplotype XIII for purposes of this analysis. Reference sequences for representative haplotypes are archived in EMBL (accession numbers Z50124-Z50140) and Encalada (1995).

Measures of within-population variation, as determined by haplotype and nucleotide diversities are presented in Table 3. The highest haplotype diversity value was observed in the Mexican population ($\hat{h}$ = 0.82), similar to the overall diversity estimate ($\hat{h}$ = 0.83). Nucleotide diversity was highest for Mexico and Aves Is., the colonies in our survey with among the lowest current population sizes (as indicated by number of nesting females per year; Appendix 1). The inverse relationship between rookery size and mtDNA diversity is apparent for other colonies (Lahanas *et al.* 1994). Overall haplotype diversity ($\hat{h}$ = 0.830) was slightly higher than that reported in an RFLP survey of loggerhead turtles (0.732; Bowen *et al.* 1993b), and comparable to that obtained from a control region sequence assessment of Atlantic hawksbills (0.849; Bass *et al.* 1996).

The relationships among the 18 mtDNA genotypes from the nine Atlantic and Mediterranean green turtle colonies are summarized in an unrooted parsimony network (Fig. 2). A striking feature of this arrangement not apparent in the earlier RFLP study (Bowen *et al.* 1992) is the phylogenetic clustering of haplotypes corresponding to geographical regions. Two major groups are distinguished: cluster A corresponds to the western Caribbean and the single Mediterranean rookery, represented by 10 haplotypes; and cluster B, includes the eastern Caribbean, South Atlantic, and West African colonies, represented by eight haplotypes. All individuals from the two groups were separated by at least five mutation steps, whereas haplotypes within either cluster differed by a maximum of five and four mutations, respectively. The distinction between these two assemblages is evident in maximum parsimony analysis for which bootstrap support was at the 100% level; in UPGMA analysis (not shown); and in neighbour-joining analysis for which bootstrap support was 83% (in 1000 replicates, Fig. 3).

Within each cluster, haplotypes were shared among proximal rookeries although at different frequencies in most cases. In cluster A, characteristic of the western Caribbean and Mediterranean regions, haplotype III was predominant, present in Florida (at 50%), Mexico (25%), Costa Rica (93%), and Aves Is. (12.5%: one individual). Mediterranean samples contained haplotypes XIII and XIV (differing by a single site change at position 436) which differed by one and two mutational changes, respectively, from haplotypes XVI and XVII, characteristic of the Mexican population. In cluster B, characteristic of the western Caribbean, South Atlantic and African

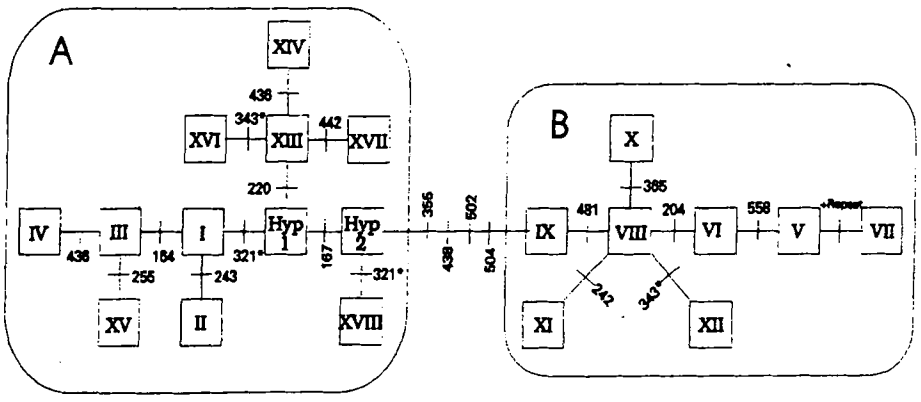

Fig. 2 Parsimony network describing interrelationships among the 18 mtDNA haplotypes. Roman numerals correspond to haplotypes in Tables 1 and 2. 'Hyp 1' and 'Hyp 2' represent hypothetical haplotypes not observed in the present survey. Capital letters (A and B) identify the two major clusters referred to in the text. Mutation site numbers are indicated along branches, and asterisks beside sites 321 and 343 denote instances in which homoplasy is assumed.

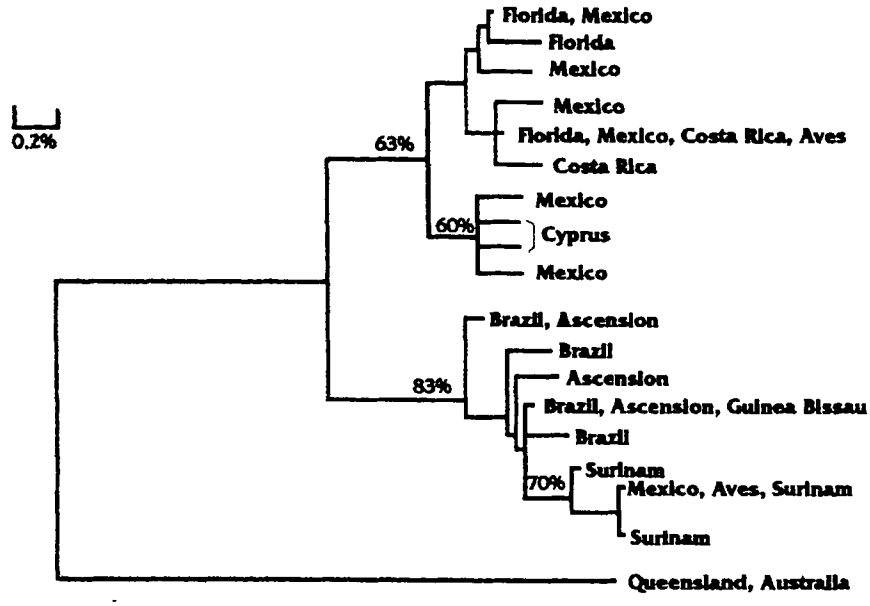

Fig. 3 Neighbour-joining tree for Atlantic green turtles based on mtDNA control region sequences, rooted using an individual from Queensland, Australia as an outgroup. The numbers at specific nodes represent the percentage recovery of the particular node in 1000 bootstrap replications. Only those percentages greater than 60 have been reported.

rookeries, haplotype VIII was predominant among samples from Guinea Bissau (100%), Brazil (50%), and Ascension Island (80%). Haplotype V (also in cluster B) was observed at high frequency in the western Caribbean rookeries at Aves Is. (87.5%) and Surinam (87%). With the exception of Cyprus, all populations shared haplotypes with at least one other colony, typically a nesting population within the same phylogenetic cluster. Exceptions to this phylogeographic pattern were single individuals in the Mexican and Aves Is. populations which were assigned to cluster B and A, respectively.

Despite the cases of haplotype sharing described above, haplotype frequencies were significantly different in 33 of 36 pairwise comparisons of nesting populations, indicating isolation or demographic independence among the colonies (Table 4). The colonies at Mexico and Florida, Surinam and Aves Is., and Ascension Is. and Guinea Bissau were not significantly different. Notably, these three pairs of colonies are in geographical proximity to each other.

In general, estimates of *Nm* lower than 1–4 indicate that gene flow between rookeries is too low to prevent geographically isolated colonies from diverging as a result of genetic drift (Birky *et al.* 1983; Slatkin 1987). *Nm* values greater than 1–4 indicate that levels of gene flow are sufficient to maintain populations at relatively homogeneous genotype frequencies (Slatkin 1987). In this study, estimates of interpopulation gene flow (*Nm*) using Takahata & Palumbi's (1985) *Gst* estimator ranged from 0.017–2.5 migrants per generation, but most values were less than 1.0, suggesting little interrookery gene flow via females (Table 4). The mean migration estimate among Atlantic and Mediterranean colonies based on the private allele approach is $Nm \approx 0.64$.

Comparison of the Atlantic data set to the Pacific outgroup produced a sequence divergence estimate of $p = 0.044$. Direct comparison of the restriction-site data from Bowen *et al.* (1992) to the control region sequencing data revealed an approximate sixfold increase in sequence divergence provided by the sequencing data (ratio of mean percentage sequence divergence: 4.4/0.7). The D-loop data set also produced higher resolution in terms of number of haplotypes detected. We observed 14 control region haplotypes in samples which contained eight RFLP haplotypes (Mexico, with four haplotypes, was not included in the RFLP analysis).

Discussion

The nesting beaches sampled in this study represent most of the major reproductive aggregates of green turtles in the Atlantic basin and Mediterranean Sea. Together, they comprise over 20 000 nesting females per year (see Appendix 1). Green turtle nesting habitat is widespread over the tropical and warm temperate region, and tagging data indicate that movement of breeding females between geographically separate nesting colonies is extremely rare (Meylan 1982). This observation is consistent with the significant differences in mtDNA genotype frequencies between most pairs of surveyed populations (Table 4). Only three pairs of rookeries could not be differentiated in the present study. This can be interpreted as continuous exchange between pairs of populations or as the result of recent isolation. The overall pattern of strong population structuring may be interpreted as evidence for the latter explanation (see Norman *et al.* 1994). However, instances of haplotype sharing between the two primary phylogeo-

Table 4 Below diagonal: pair-wise haplotype frequency comparisons based on χ^2 statistics using the Monte Carlo randomization method and the sequential Bonferroni procedure. Chi-square and *P*-values appear in bold for three population pairs which did not differentiate (NS, non-significant). Above diagonal: migration rates (*Nm*) based on *Gst* estimator. The migration estimate by private alleles method is *Nm* = 0.64 migrants/generation

Population	Florida	Mexico	Costa Rica	Aves	Surinam	Brazil	Ascension	Guinea Bissau	Cyprus
Florida	—	2.3	0.41	0.42	0.44	0.27	0.39	0.57	0.14
Mexico	**12.51 P=0.031 NS**	—	0.35	0.29	0.28	0.57	0.31	0.16	0.23
Costa Rica	11.70 *P*=0.002	19.96 *P*~0.000	—	0.063	0.057	0.16	0.073	0.017	0.047
Aves	27.08 *P*~0.000	19.63 *P*=0.001	18.89 *P*~0.000	—	2.5	0.19	0.099	0.032	0.065
Surinam	39.00 *P*~0.000	31.21 *P*~0.000	30.00 *P*~0.000	**2.94 P=0.761 NS**	—	0.20	0.10	0.035	0.069
Brazil	40.00 *P*~0.000	36.00 *P*~0.000	31.00 *P*~0.000	24.00 *P*~0.000	31.00 *P*~0.000	—	1.09	0.43	0.17
Ascension	44.00 *P*~0.000	40.00 *P*~0.000	35.00 *P*~0.000	28.00 *P*~0.000	35.00 *P*~0.000	11.03 *P*=0.006	—	1.13	0.089
Guinea Bissau	43.00 *P*~0.000	39.00 *P*~0.000	34.00 *P*~0.000	27.00 *P*~0.000	34.00 *P*~0.000	12.32 *P*~0.000	**4.23 P=0.163 NS**	—	0.026
Cyprus	34.00 *P*~0.000	30.00 *P*~0.000	25.00 *P*~0.000	18.00 *P*~0.000	25.00 *P*~0.000	26.00 *P*~0.000	30.00 *P*~0.000	29.00 *P*~0.000	—

graphic groups (by single individuals from Mexico and Aves Is.) might also indicate a low level of genetic exchange between different geographical regions, illustrating the dynamic structure of green turtle rookeries over evolutionary timescales. A strong maternal population structure is not unique to *C. mydas*, as indicated by comparable surveys of hawksbill turtle (*Eretmochelys imbricata*), and loggerhead turtle (*Caretta caretta*) nesting populations (Bowen *et al.* 1993b; Broderick *et al.* 1994; Bass *et al.* 1996). Surveys of other marine vertebrates with nearly unlimited migratory potential also demonstrate maternally directed site fidelity (Baker *et al.* 1994).

The demographic independence of green turtle nesting populations is reflected in the geographical structure of mtDNA lineages in the Atlantic–Mediterranean system. Two distinctive evolutionary lineages were observed corresponding to the western Caribbean and Mediterranean rookeries (cluster A), and the eastern Caribbean, South Atlantic and West African rookeries (cluster B; Fig. 2; Fig. 3). Based on a provisional RFLP molecular clock of 0.2–0.4% divergence per million years for the Testudines (Avise *et al.* 1992; Bowen *et al.* 1993a), the observed sixfold increase in control region sequence divergence yields an evolutionary rate of about 0.012–0.024 substitutions/site per million years. This estimate places the divergence of Atlantic green turtle lineages (clusters A and B) at ≈ 0.3–0.7 Myr, and the divergence from the Pacific outgroup at ≈ 1.8–3.7 Myr.

The gene genealogy generated in this study allows us to distinguish between two possible models for the distribution of mtDNA lineages in the Atlantic basin, that which explains the disjunct distribution of organisms in terms of formerly continuous habitats sundered by geological or climatic changes (vicariant scenarios), and that which emphasizes the ability of organisms to occasionally transplant far beyond the boundaries of their historical distribution (dispersal scenarios). A vicariant scenario for the origin of the Ascension Island rookery has been already refuted with mtDNA data, which indicate a recent colonization event for this remote mid-Atlantic island

(Bowen *et al.* 1989). Here we consider the geological and climatic history of the Pleistocene, together with the observed mtDNA phylogeny, to determine how these factors might explain the observed phylogeographic pattern in Atlantic green turtles.

The data presented here and in Bowen *et al.* (1992) indicate that green turtles in the Atlantic and Pacific Oceans were probably isolated by the early Pleistocene (about 2.0 Myr). Unlike the loggerhead turtle, which can be found nesting along temperate coastlines, the green turtle is restricted to tropical and subtropical ranges. At the time of maximum glacial episodes, the advance of continental ice sheets and the consequent drop in sea level [≈ 100 m below present surface levels (Bowen 1978)] probably reduced the range of suitable green turtle foraging and nesting habitat to a narrow tropical margin. Indeed, climatic records for the Wisconsin glaciation (the last glacial maxima: 18 000 years ago), indicate that sea ice extended at least 10 degrees of latitude closer to the equator than it does today (Gates 1993). Under these conditions, characterized by a combination of colder temperatures and elevated aridity, requirements for suitable green turtle habitat were probably not met at the contemporary Atlantic green turtle nesting sites of higher latitudes (Florida, Yucatan and Cyprus, for example).

In contrast, the fossil record of the Early Pleistocene shows that glacial conditions had little effect on the fauna at low latitudes, with stable conditions maintained in equatorial regions (Cooke 1972; Bowen 1978). Thus, equatorial regions may have served as green turtle 'refugia' during glacial maxima, with subsequent colonizations into higher latitudes during warmer interglacial periods (Fig. 4). The central position of haplotype VIII in the parsimony network (Fig. 2) makes this sequence a possible candidate for the closest relative to an ancestral Atlantic haplotype. Notably, haplotype VIII represents the hub from which other Atlantic lineages 'radiate', and it is the most common and widespread haplotype in equatorial rookeries (Fig. 4A), occurring in 43 of the 55 individuals assayed.

Within the equatorial region, precursors of haplotypes V, VI, and VII may have colonized the north-eastern coast of South America (into present day Surinam and other Guyana colonies, Fig. 4). Moll (1983) suggested that at times of decreased sea levels, foraging resources available to Brazilian and Ascension turtles fractioned to the north and south of the Brazilian bulge, and thus prompted colonization by turtles to these areas. Although Moll's vicariant scenario can account for the colonization of northern feeding areas during glacial maxima, the gradual dislocation of feeding grounds is not likely to have accounted for the colonization of distant rookeries at higher latitudes. Thus, long-distance dispersal of haplotypes during glacial retreats (with the consequent rise of temperatures and availability of suitable feeding and nesting conditions), is strongly implicated for the colonization of western Caribbean and Mediterranean nesting colonies (Fig. 4, B and C). Precursors to haplotypes XIII and XIV probably colonized the Mediterranean Sea during the current interglacial period, as indicated by climate records and the close relationship between Caribbean and Cyprus haplotypes (Fig. 4C). Although the most recent glacial ice sheet began to retreat ≈ 18 000 years ago, the Younger Dryas Event represents a global cooling phenomenon between 11

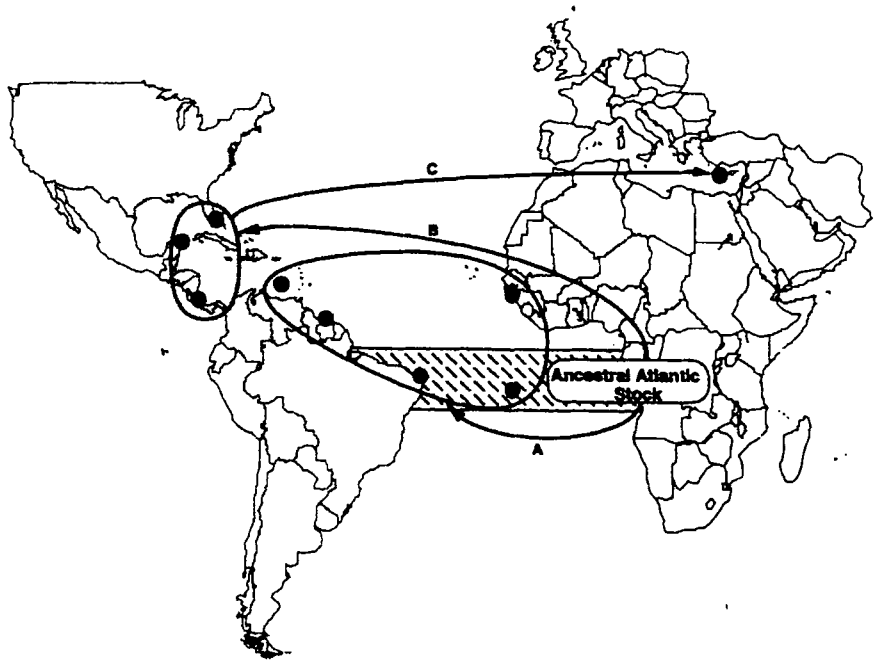

Fig. 4 Present distribution of haplotype groups, and possible colonization events (arrows) of green turtle mtDNA genotypes in the Atlantic and Mediterranean region. The location of presumed Quaternary green turtle refugia (hatched), is shown along stable tropical latitudes. Long-distance colonization events are indicated as follows: A, from an ancestral Atlantic stock to present day Ascension, Brazil, Guinea Bissau, Surinam and Aves Is. colonies (cluster B haplotypes); B, from ancestral Atlantic stock to western Caribbean (cluster A haplotypes); and C, from western Caribbean colonies to the Mediterranean.

and 10 000 years ago (Engstrom *et al.* 1990; Kudrass *et al.* 1991; Gates 1993). Thus, colonization events into the northwestern Caribbean and Mediterranean most likely occurred within the last 10 000 years.

The marked population structure of Atlantic green turtle rookeries suggests that despite this creature's high vagility, historical and life-history factors have been important in initiating and maintaining population genetic structure and contemporary restrictions on gene flow among populations. Over ecological timescales, nest-site fidelity appears to be a primary influence on population structure and demography, as indicated by the significant haplotype frequency shifts observed among Atlantic nesting colonies. Over evolutionary time scales, as green turtle habitat expands and contracts with glacial cycles, departures from nest site fidelity are sufficient to colonize newly opened habitat at high latitudes, even when that habitat is thousands of kilometres from glacial refugia. The balance between nest site fidelity and long distance dispersal (as illustrated by mtDNA data), is a key to understanding the phylogeography of Atlantic green turtles.

Finally, the observed haplotype frequency shifts between nesting colonies provide an appropriate basis for resolving the composition of feeding cohorts (see Bowen 1995; Norman *et al.* 1994). Given the composite nature of the foraging areas (which sustain individuals from diverse rookeries), it is imperative to determine the stock composition of such areas in order to establish conservation and management plans which incorporate the entire life history of this species. The survey described herein, including control region sequences from almost all major Atlantic nesting colonies, provides a foundation for the development of both regional and rookery-specific genetic markers for the assessment of feeding ground composition (P.N. Lahanas *et al.* unpublished data). Similar markers have been developed for the identification of management units of Indo-Pacific green turtles (Norman *et al.* 1994), and feeding ground composition of Indo-Pacific hawksbill turtles (Broderick *et al.* 1994), and Atlantic hawksbills (Bowen *et al.* 1996). Furthermore, a more recent use of these data has been as a molecular tool with population-specific diagnostic power in the identification of the origin of confiscated or stranded turtles in forensic studies (Encalada *et al.* 1994). The genetic markers defined in this study will likely have additional applications in investigations of green turtle natural history and conservation.

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Appendix 1 Sample locations, sample sizes and estimate sizes of nine green turtle nesting beaches

Nesting beach location	Sample size (n)	Nesting population size (females/year)	Reference
Hutchinson Is, Florida	24	a few hundred	Conley & hoffman 1987
Quintana Roo, Mexico	20	100 – 400	Zurita *et al.* 1993
Tortuguero, Costa Rica	15	5000 – 23000	Carr *et al.* 1978
Aves Is, Venezuela	8	300 – 500	Sole & Medina 1989
Matapica, Surinam	15	a few thousand	Schulz 1982
Atol das Rocas, Brazil	16	50 – 100	Bowen *et al.* 1992
Ascension Is, UK	20	1600 – 3000	Mortimer & Carr 1987
Pailoa, Guinea Bissau	19	about 400	Bowen *et al.* 1992
Lara Bay, Cyprus	10	fewer than 100	Demetropoulos & Hadjichristophorou 1992