Conservation Genetics (2006) 7:353–369
DOI 10.1007/s10592-005-9047-z

Mitochondrial DNA diversity and phylogeography of endangered green turtle (*Chelonia mydas*) populations in Africa

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Received 22 February 2005; accepted 28 July 2005

Key words: Africa, control region haplotypes, population structure, sea turtles, phylogeography

Abstract

We analysed the genetic structure of seven nesting sites of the endangered green turtle (*Chelonia mydas*) in Africa using mitochondrial DNA control region sequences. Tissue samples were collected from 188 nesting females at six sites in West Africa and one in the Indian Ocean. A 488 bp fragment of the control region revealed 14 different haplotypes, 10 of which are previously undescribed. The most common haplotype (CM8) was observed in 157 individuals. All other haplotypes were closely related, except two divergent lineages: CM38, removed by four substitutions, and the three Indian Ocean haplotypes, distinguished by 31 substitutions. Significant differences in haplotype and nucleotide diversity were observed between Atlantic rookeries and among ocean basins. Analysis of molecular variance revealed high levels of differentiation between the Atlantic and the Indian Ocean populations but a much shallower Atlantic substructuring. Green turtle population genetic structure is thought to have been shaped by a dynamic succession of extinction and recolonisation of rookeries, by natal homing and occasional breakdown in nest-site fidelity. Mismatch distributions of pairwise differences between haplotypes at each rookery were found to be consistent with recent population expansion. We argue that demographic histories can be explained by scenarios at several temporal scales, including geological events, sea level fluctuations and more recent patterns of exploitation. We discuss management and conservation implications of our results for these threatened populations, identifying two ESUs (one in the Atlantic and one in the Indian ocean) and three MUs within the Atlantic.

Introduction

Green turtles (*Chelonia mydas*) are marine turtles with a worldwide tropical and subtropical distribution and are endangered across much of their range. They nest on sandy beaches and exhibit natal homing behaviour (Meylan et al. 1990). Adults are herbivorous, feeding primarily on algae and seagrasses, and undertake extensive migrations between breeding and foraging grounds, which may span thousands of kilometres. Hatchlings spend several years in pelagic environments before recruiting to neritic habitats as juveniles. Like other long-lived marine species, green turtles are difficult to study during their marine life stages and population structure and distribution are not fully understood.

Recent evidence has shown that important green turtle nesting and feeding populations present along the Atlantic coast of Africa are threatened by various factors (UNEP/CMS 2000; Fretey 2001; Formia and Fretey unpublished). The high demand for turtle meat and products such as carapaces, eggs and oil, has led to heavy exploitation. Individuals of all sizes are captured intentionally and incidentally in fishing nets and nesting females are taken from the beaches. In addition, extensive areas suffer from habitat degradation due to coastal development, erosion, sand mining, trawling, pollution and oil drilling. Due to their long generation times and highly migratory habits, green turtle populations are subject to threats over wide geographic ranges; protection of a nesting habitat may be rendered meaningless by mortality of the same population in unprotected migration routes, developmental and feeding habitat, or vice versa.

Habitat use, philopatry, mating behaviour and migratory patterns play an important role in determining population structure, and molecular techniques have proved effective in gaining insights in these behaviours which are difficult to observe in sea turtles (e.g. Meylan et al. 1990; Karl et al. 1992; Allard et al. 1994; Norman et al. 1994; Bowen et al. 1995, 1996; Bass et al. 1996; Dutton 1996b; FitzSimmons et al. 1997; Laurent et al. 1998; Roberts et al. 2004). Due to natal homing behaviour, green turtle populations are subdivided into geographically and genetically distinct nesting assemblages. Mitochondrial DNA (mtDNA) has been shown to be the most appropriate molecular marker to resolve this matrilineal structure, providing a high degree of resolution in addressing regional-scale questions (Bowen et al. 1992; Allard et al. 1994; Lahanas et al. 1994; Norman et al. 1994; Encalada et al. 1996; FitzSimmons et al. 1997). Nesting populations may aggregate in feeding grounds, developmental habitat or migratory routes (Bass et al. 1998; Lahanas et al. 1998; Bass and Witzell 2000). Determination of stock boundaries and assessment of mixed stock composition are priorities of current research for this species; the power and resolution of mixed stock analysis is increased by accurate genetic data on potential contributor populations (Bolker et al. 2003). To date, such information has been lacking for green turtle populations in West Africa and the Indian Ocean. In addition, management and conservation efforts may be enhanced by better understanding population ranges and identifying which rookeries are affected by threats away from the nesting beach.

Green turtles often nest on isolated oceanic islands lacking mammalian predators (Mortimer 1995). This study focuses on island rookeries located in the eastern Atlantic Ocean and in the southwestern Indian Ocean. It encompasses the islands of Poilão (Bijagos Archipelago, Guinea Bissau), Ascension (UK) (located approximately mid-way between the coasts of South America and Africa), Bioko (Equatorial Guinea), São Tome and Principe. Bioko, São Tome and Principe are part of a volcanic island chain bisecting the Gulf of Guinea. Additional sporadic nesting has been reported along the entire West African coast but much of it unconfirmed or limited in numbers (Formia and Fretey unpublished). Nesting in the southwestern Indian Ocean includes several important island rookeries: Europa, Tromelin, Mayotte, Seychelles and Comoros, as well as low density nesting along the coasts of Tanzania, Mozambique and South Africa. The Comoros sample studied here was collected on Moheli Island, located approximately two-thirds of the way between northern Madagascar and northern Mozambique in the Mozambique Channel.

We analysed mtDNA control region sequences of significant green turtle nesting populations with the aim of assisting conservation efforts. Standardising the region sequenced allowed comparison with other studies in the Atlantic and worldwide. We present data on the haplotype frequency distribution of previously unsampled or under-sampled nesting populations, extending earlier phylogeographic descriptions in the Atlantic (Allard et al. 1994; Lahanas et al. 1994, 1998; Encalada et al. 1996) to the coast of Africa. We also evaluate inter-oceanic divergence, assess rookery demographic history and propose scenarios explaining the observed patterns of diversity at both contemporary and evolutionary timescales.

Methods

Samples were collected from seven island nesting populations (Figure 1): 51 from Poilão (Guinea Bissau), 50 from Ascension Island (random subset of samples used in Formia et al. unpublished), 51

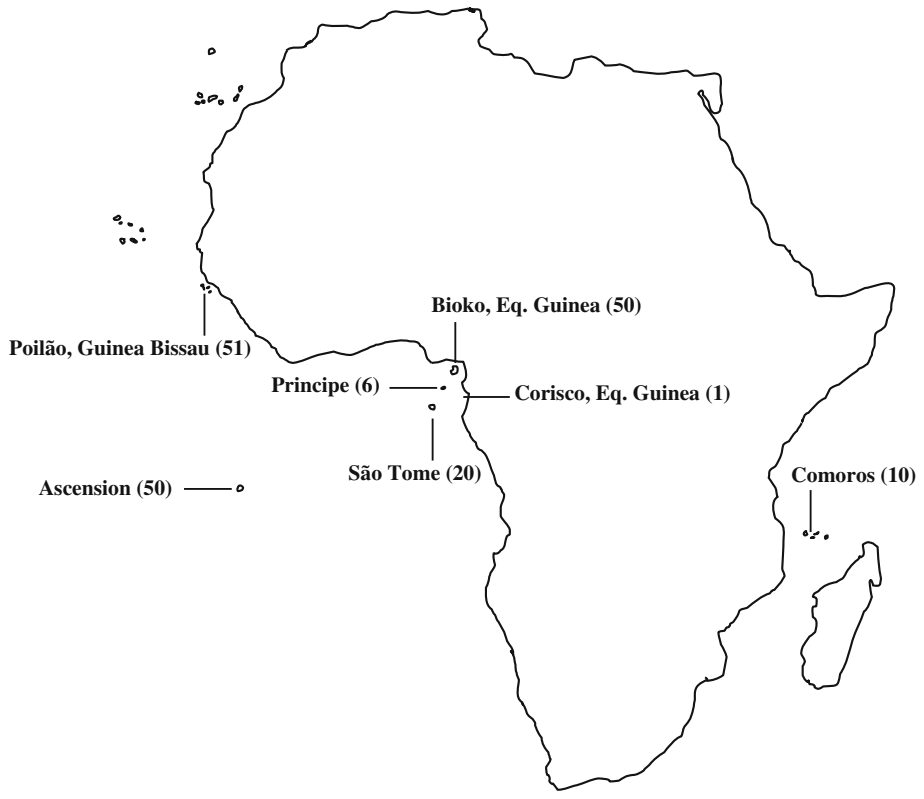

Figure 1. Sample sizes and sampling sites.

from Equatorial Guinea (50 from Bioko and a single sample from Corisco Island), 20 from São Tome, six from Principe (São Tome and Principe) and 10 from Moheli (Comoros). The size of these rookeries is approximately 1500 nesting females per year in Poilão (calculated from Catry et al. 2002), 3000–5000 in Ascension (Godley et al. 2001), 400–600 in Bioko (Tomás et al. 2000), 80–100 in São Tome (Dontaine unpublished), 75–100 in Principe (Dontaine unpublished), 0–10 in Corisco (Formia unpublished) and 5000 in Comoros (Ahamada pers. commun.). Corisco Bay is a green turtle foraging ground (Formia 1999) where nesting is considered infrequent. Due to sample size this site was excluded from most of the analysis. Rookery sizes were estimated based on number of nests, assuming three clutches per female per season.

Sampling took place during the following nesting seasons: Poilão (2001), Ascension (1998–1999, 1999–2000, 2000–2001), Bioko (1998–1999, 1999–2000), Corisco (1999–2000), Principe (1998–1999, 1999–2000), São Tome (1998–1999, 1999–2000) and Comoros (2001). The sample collection protocol was modified from Dutton (1996a). Samples from live animals were fresh skin biopsies, approximately 5 mm in diameter, taken from the neck or anterior flipper of nesting females during the oviposition phase to cause minimal disturbance. Three samples from Bioko were collected from dry tissue from the inner surface of old carapaces. One sample from Comoros, 42 samples from Poilão and 22 samples from Ascension were collected from a representative dead hatchling from nests emerging within a 12-day inter-nesting interval. All samples were stored at ambient temperature in 20% w/v DMSO in saturated NaCl.

Samples were finely chopped and digested overnight with proteinase-K at 37 °C. DNA was then extracted using a standard phenol/chloroform protocol (Milligan 1998) followed by ethanol precipitation, or using a modification of the protocol by Allen et al. (1998). DNA was stored in TE buffer at −20 °C. A 488 bp mtDNA control region fragment was amplified using primers LTCM1 and HDCM1 described by Allard et al. (1994).

Polymerase chain reaction (PCR) (1.5 mM $MgCl_2$, 1× PCR buffer, 200 μM each dNTP, 0.5 μM each primer, 0.5 U Gibco BRL *Taq*, 1 μl template DNA and H_2O to a total volume of 10 μl) was carried out under the following conditions: 3 min at 94 °C, 35 cycles of 45 s at 94 °C, 30 s at 55 °C and 1.5 min at 72 °C, followed by 10 min at 72 °C, using a GeneAmp PCR System 9700 (Perkin Elmer). The three carapace samples and seven of the Comoros samples did not amplify successfully and were analysed using two primer pairs (LTCM1–HDCM1.1 and LTCM1.1–HDCM1) designed to target shorter fragments of approximately 300 and 220 bp which, when combined, yielded the entire sequence. The new primers were designed using the program Primer3 (Rozen and Skaletsky 1996, 1997, 1998): 5′-CAA CCA TGA ATA TCG TCA CAG-3′ (LTCM1.1) and 5′-AGG GTT GCT TAT TTC TCG TG-3′(HDCM1.1). PCR conditions were modified for these 10 samples to include 0.8 μl acetylated 1× BSA; a second PCR, using the first as template, was used to obtain higher product concentration. Negative controls were used to test for contamination. PCR products were cleaned using a Geneclean Turbo Kit (Bio 101), then sequenced in both directions with the ABI Prism Big Dye Terminator kit Versions 2 or 3 (Applied Biosystems) and analysed with an Applied Biosystems model 3100 sequencer. Sequences were then aligned and edited using Sequencher 3.1.2 (Gene Codes Corporation).

Haplotype ($\hat{h}$) and nucleotide diversity (π) were calculated for each site using Arlequin V. 2.0 (Schneider et al. 2000). TCS V. 1.13 (Clement et al. 2000) was used to assess relationships among haplotypes through a matrix of number of substitutions between sequences. In turn, this matrix was used by Minspnet (Excoffier and Smouse 1994) to design a minimum spanning network illustrating equally parsimonious haplotype trees.

Pairwise comparisons of rookeries and a nested analysis of molecular variance (AMOVA) were used to determine the partitioning of variation within and among rookeries, and within and among ocean basins, using Arlequin ver 2.0 (Schneider et al. 2000). AMOVA calculates Φ-statistics using a model of genetic distance and a gamma parameter estimate (Excoffier et al. 1992), as well as conventional *F*-statistics under a model of equal genetic distance which are analogous to θ (Weir and Cockerham 1984). The gene flow between population pairs was calculated using the expression $N_m = 0.5[(1/F_{ST})-1]$ (Takahata and Palumbi 1985). Significance values were obtained from a minimum of 1000 permutations.

Mismatch distributions were calculated for each rookery using Arlequin (Schneider et al. 2000). This analysis describes the distribution of pairwise nucleotide differences among DNA sequences, estimating τ (expansion time), θ_0 and θ_1 (mutation parameters) based on a model of sudden population expansion (Rogers and Harpending 1992; Harpending et al. 1998; Schneider and Excoffier 1999), and thus allowing an estimation of the time since establishment. The validity of the estimated mismatch parameters was tested using Arlequin's sum of square deviations (SSD) test of goodness of fit, comparing observed and expected mismatch distributions (Schneider and Excoffier 1999). The following equations were then used to calculate t (time in generations since expansion) and N_0 (effective population size before expansion): $t = \tau/2u$, where $u = 2\ \mu k$, k is the number of nucleotides in the sequences being compared and μ is the mutation rate; $N_0 = \theta_0/2u$ (Rogers and Harpending 1992). We used the program r8s to estimate mutation rates from our data (Sanderson 2003), applying the semi-parametric penalized likelihood method. We assumed a generation time of 47 years (Seminoff et al. 2002). We did not estimate the population size after expansion (N_1), as it is not believed to be adequately recovered from the mismatch distribution (Schneider and Excoffier 1999). We calculated 95% confidence intervals for all parameters with 100 replicates using Arlequin's parametric bootstrap approach.

Results

Haplotype distribution and genetic diversity

Sequence analysis of the 188 samples revealed 47 variable positions defining 14 haplotypes (Table 1), 10 of which are previously undescribed (CM35–CM39, CM45–CM46, IND1–IND3). The 178 sequences from the five rookeries along the Atlantic coast of Africa exhibited 11 haplotypes, seven of which are previously undescribed (Table 2). All haplotypes found in the Indian Ocean rookery are previously undescribed (IND1–

Table 1. Polymorphic sites defining 14 haplotypes, all previously undescribed except CM5, 6, 8 and 24

Haplotype	Base position
	0 1 1 3 4 4
	0 2 9 6 0 8
	2 8 9 2 7 2
	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
	5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 6
	5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 8 8 8 8 8 8 8 8 8 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 0
	2 2 3 3 5 8 1 1 3 5 7 8 8 8 9 9 0 0 2 3 4 5 0 1 1 7 8 8 8 8 9 0 0 2 2 2 2 2 4 4 4 4 5 5 5 6 0
	4 5 8 9 3 8 0 3 1 0 7 2 4 8 2 3 8 9 1 0 7 0 1 1 5 4 2 4 6 8 0 1 2 5 6 7 8 9 2 5 8 9 0 4 5 6 4
CM5	A T – – C G C G A A A A C G T T A C T G C A G C A A C A C G G T T A G C T T C G G C A T T C G
CM6	· · – – · A
CM8	· · – – · · · · · G · A
CM24	· · – – · · · · · G · A · · · · · · A
CM35	· · – – · · · · · · · · · A · A
CM36	· · – – · · · · · G · G · · A
CM37	· · – – T · · · · G · A
CM38	· · – – · · · · · G · · · · · · · · C · · G · · · · · · · · · C · · · A · · · · · · · · · · A
CM39	· · – – · T · · · · · · · · · · · · · · · · · · · A
CM45	· · – – · · · · · G · C · · · · · · · · · · · · · A
CM46	· · – – · · · · · G · · · · · · · · · · · · · · · · T · · · · · · · · · · · · · · · · · · · A
IND1	G G G C · A T A C G G T – · C C · T · A T · T T G G T G T A A C C G T · · · · A · T · · C T A
IND2	G G G C · A T A C G G T – · C C G T · A T · T T G G T G T A A C C G T · · · · A · T · · C T A
IND3	G G G C · A T A C G G T – · C C · T · A T · T T G G T G T A A C C G T · · · · A · T · · · T A
NRC	? T T · · · · T · · · C G T T C C T A A · G · · T ?

Polymorphisms include 100 transitions and 19 transversions (underlined). Base positions are shown based on a scale of 0–488 (top row) and on alignment with the complete green turtle mtDNA sequence of 16,497 nucleotides (Kumazawa and Nishida 1999; accession number ABO12104). CM5 is taken as the reference sequence; dots (·) indicate equal nucleotides, dashes (–) indicate indels, question marks (?) are unknown bases. The sequence NRC is only 226 bp and is not considered a haplotype.

IND3) (Table 2). The most commonly occurring haplotype was CM8 (83.5%), found in all Atlantic populations in different frequencies. The second most common haplotype (CM6) was only found in 4.8% of the samples. Ascension, São Tome and Comoros possessed unique haplotypes; 11 haplotypes only occurred at a single site. However, unique haplotypes were rare, only occurring in one or two individuals (eight haplotypes only occurred in one individual, three occurred in two). In addition, two identical partial sequences of 226 bp (due to the failure of the amplification by LTCM1–HDCM1.1) were temporarily described as NRC from Comoros but were not assigned a haplotype name. These sequences were included in all subsequent analysis except the minimum spanning network. Reference sequences for new haplotypes are in GenBank (accession numbers AY044848–AY044852 and AF529026–AF529030) and on the website of the Archie Carr Center for Sea Turtle Research (ACCSTR).

Haplotype diversity (Table 3) at each Atlantic rookery and at all Atlantic rookeries combined (n = 178) was found to be substantially lower than within a single Indian Ocean nesting population (n = 10). Likewise, nucleotide diversity (Table 3) in the Indian Ocean rookery was almost 40 times higher than in the Atlantic. Diversity in Atlantic rookeries was highly variable, ranging from 0.000 in Poilão (fixed for haplotype CM8) to São Tome's 0.584 ($\hat{h}$) and 0.003 (π), and did not appear to be affected by sample size. Values of diversity in the eastern Atlantic rookeries were comparable to those reported by Encalada et al. (1996) for the western Atlantic and Caribbean.

The minimum spanning network (Figure 2) showed that most haplotypes appeared to be related and removed by one substitution from their nearest neighbour. They were found to be

Table 2. Haplotype distribution by rookery, available sample sizes and number of haplotypes

Rookery/Haplotype	Poilão	Ascension	Bioko	Corisco	Principe	São Tome	Comoros	n
CM5						1		1
CM6		3	5			1		9
CM8	51	43	45	1	4	13		157
CM24		1						1
CM35						1		1
CM36					2	1		3
CM37						1		1
CM38						2		2
CM39		1						1
CM45		1						1
CM46		1						1
IND1							5	5
IND2							1	1
IND3							2	2
NRC							2	2
n	51	50	50	1	6	20	10	188
Haplotypes	1	6	2	1	2	7	3	

grouped around a central sequence represented by haplotype CM8. However, two divergent branches occurred: (a) CM38 is a novel haplotype separated by four substitutions from CM8 and found in two individuals in São Tome; (b) the highly divergent haplotypes IND1–3 from Comoros were separated by at least 31 substitutions from their nearest neighbour. These haplotypes also showed a two base insertion and a deletion at one position with respect to the rest.

Table 3. Haplotype and nucleotide diversities for all rookeries ± SD (except Corisco, due to sample size)

Rookery	Haplotype diversity ($\hat{h}$)	Nucleotide diversity (π)
Poilão	0.000 ± 0.000	0.0000 ± 0.0000
Ascension	0.260 ± 0.081	0.0006 ± 0.0007
Bioko	0.184 ± 0.068	0.0004 ± 0.0006
Corisco	n.a.	n.a.
Principe	0.533 ± 0.172	0.0011 ± 0.0012
São Tome	0.584 ± 0.127	0.0030 ± 0.0021
Atlantic Overall	0.220 ± 0.041	0.0007 ± 0.0008
Comoros	0.733 ± 0.120	0.0261 ± 0.0154
Overall	0.300 ± 0.044	0.0065 ± 0.0038

Also shown are overall values for Atlantic sequences and for all sequences combined. No gamma correction was applied to nucleotide diversity calculations using the Kimura 2-parameters method in Arlequin (Schneider et al. 2000).

The partial sequence NRC also represented a divergent branch (not shown in the minimum spanning network), distinguished by at least 14 substitutions from IND3. All haplotypes from the Atlantic grouped separately from those sampled in the Indian Ocean. The unresolved ambiguity between haplotypes CM8, CM6, CM46 and CM39 (dashed line in Figure 2) is likely to be a consequence of homoplasy, convergent site changes or reverse mutations at the two most variable sites (positions 15650 and 15882). In addition, three equally parsimonious solutions were possible connecting the Atlantic and Indian Ocean haplogroups (dashed lines in Figure 2).

Haplotype distribution in each of the Atlantic rookeries was illustrated on a reduced portion of the minimum spanning network (Figure 3). Notably, Ascension and São Tome shared the common haplotypes CM8 and CM6, but none of nine other haplotypes were found in both rookeries. Between them, these two rookeries exhibited all of the Atlantic haplotypes, while Poilão, Bioko and Principe generally exhibited fewer and more common haplotypes.

Population structure

Analysis of molecular variance was used to examine differentiation among ocean basins,

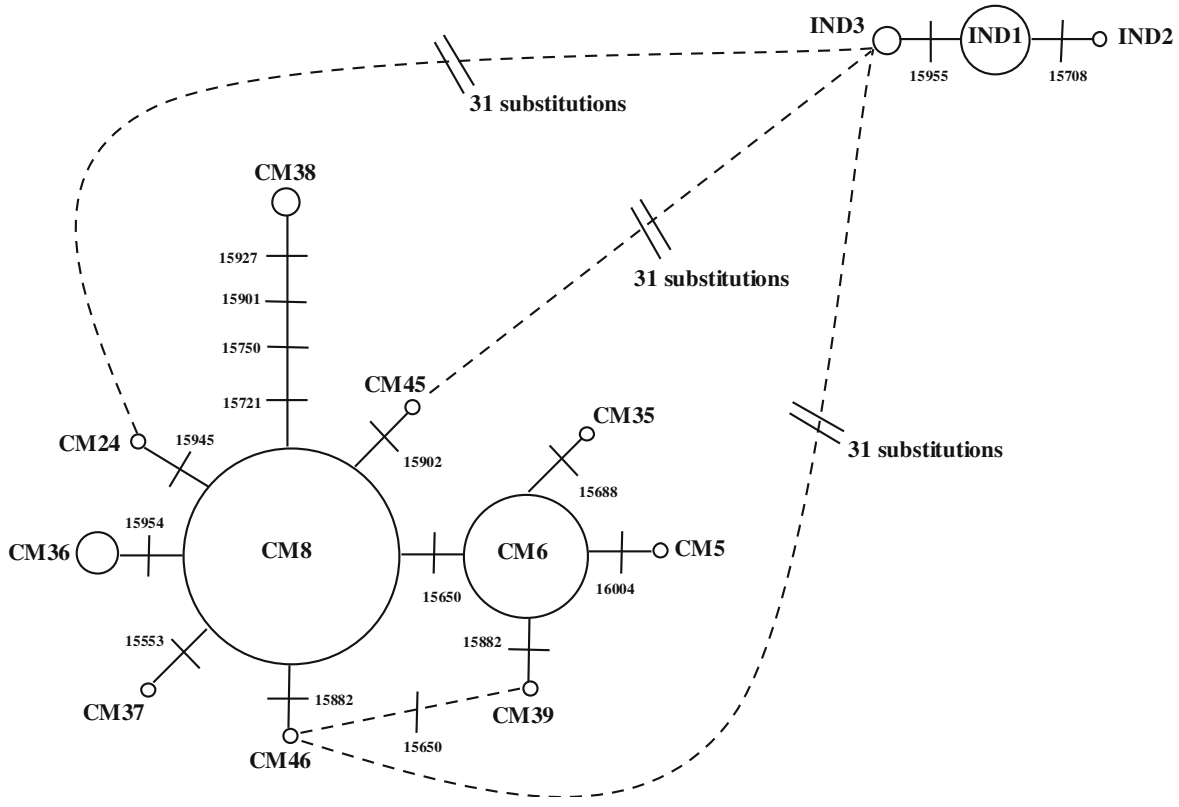

Figure 2. Minimum spanning network of the haplotypes. Haplotypes and substitution sites correspond to Table 1. The size of haplotypes is approximately proportional to their frequency in the overall sample set, except CM8, which is drawn to one-tenth of proportional size due to space constraints. Dashed lines represent ambiguous connections.

among rookeries in each basin and within rookeries (Table 4, part I). Although there is debate on whether *F*-statistics or Φ-statistics represent more realistic measures, O'Corry-Crowe et al. (1997) suggested that conventional *F*-statistics may be more accurate estimates of population subdivision when dealing with numerous haplotypes distinguished by small numbers of substitutions, and when populations are distinguished by differences in haplotype frequencies rather than clear geographic subdivision and interhaplotypic differences. In our study, this is the case within the Atlantic, but not when comparing Atlantic and Indian Ocean rookeries. With respect to the latter, it is likely that the discrepancy found between Φ_{ST} (0.9773) and F_{ST} (0.7044) can be attributed to bias in calculation methods (with genetic distance weighing more heavily than haplotypic frequencies alone), although both estimates explained the great majority of the variation as deriving from inter-oceanic differences. Given also that F_{ST} is likely to be more affected by sample size, we infer that Φ_{ST} of 0.9773 is the more realistic value of inter-oceanic differentiation.

Pairwise comparisons between populations within the Atlantic were used to determine the level of genetic differentiation among rookeries (Table 5). *F*- and Φ-statistics yielded similar results, with significant values ranging from 0.0363 to 0.6874. Population comparisons all showed significant partitioning except for: Ascension versus Bioko, and São Tome versus Principe. Better resolution might be possible with larger sample sizes from both Principe and São Tome. Pooling the samples from these two rookeries yielded slightly more significant estimates of population differentiation than for each rookery alone (Table 5). Pairwise comparisons between Comoros and individual Atlantic rookeries were all highly significant, with measures of $N_m \ll 1$.

AMOVA was performed for the Atlantic only, to determine partitioning of the variation among

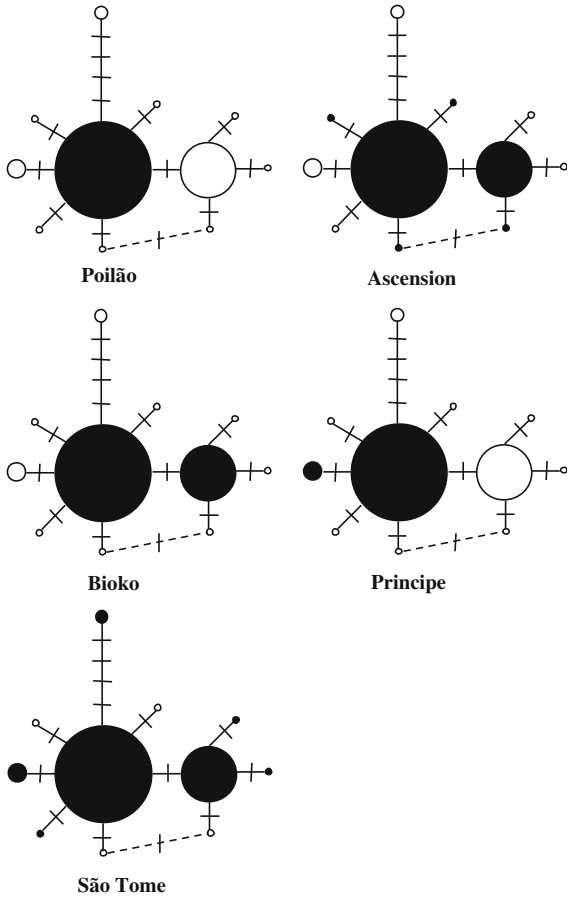

Figure 3. Haplotype diversity at each of the sampling sites in the Atlantic. Black haplotypes indicate those found within each rookery. Haplotype positions and substitution sites correspond to Figure 2.

and within rookeries (Table 4, part II). Results showed that approximately 91–94% of the variance could be accounted for by differences within populations and approximately 6–9% by differences among populations.

Mismatch distribution

Mismatch distributions and estimated parameters for Ascension, São Tome, Bioko and Comoros were all found to be compatible with the unimodal distribution model of a rapidly expanding population at $P > 0.05$ for each SSD test (Figure 4). Principe was excluded due to small sample size, while Poilão was excluded because it was fixed for a single haplotype. The mean mutation rate was estimated as 0.01751 substitutions/site per Myrs (SD = 0.00504), which was consistent with the green turtle mutation rate of 1.2–2.4% / site per Myrs derived by Encalada et al. (1996). Using this rate ($\pm$2SD) and mismatch parameters, Comoros was calculated to have the oldest average expansion of $t \approx 182{,}000$ years ago (115,000–500,000 years) and Bioko the most recent of $t \approx$ 8000 years ago (5000–22,000), while Ascension had an expansion time of approximately 88,000 years (56,000–241,000). Rather than recent expansion, a τ of zero in São Tome indicates uncertainty in the calculation process, also reflected in the large 95% confidence interval (0–5.383) and a mean value of 1.212. From this mean we estimated an expansion time of approximately 35,000 years (22,000–97,000). Ascension

Table 4. AMOVA based on two methods: A. Using a distance matrix calculated with the Kimura 2-parameter model of evolution with no gamma correction. B. Using conventional *F*-statistics from haplotype frequencies only. Part I shows the analysis of variance for all rookeries in the Atlantic and Indian Oceans. Part II is restricted to the Atlantic Ocean populations of Poilão, Ascension, Bioko, São Tome and Principe

		A. Using genetic distance (% Variance)	B. Using haplotype frequencies (% Variance)
I	Among oceans	97.68	68.05
	Among populations within oceans	0.05	2.39
	Within populations	2.27	29.56
	Φ_{ST}/F_{ST}	0.9773 ($P = 0.0000$)	0.7044 ($P = 0.0000$)
	Nm	0.0116	0.2098
II	Among populations	6.43	8.68
	Within populations	93.57	91.32
	Φ_{ST}/F_{ST}	0.0643 ($P = 0.0013$)	0.08684 ($P = 0.0010$)
	Nm	7.2760	5.2577

Table 5. Pairwise comparisons between Atlantic rookeries (excluding Corisco)

	Plo	Asc	Bk	Pr	ST	ST + Pr
Plo	–	13.2775 6.5442	5.5435 5.5435	0.2274 0.2274	3.3729 1.2023	4.9219 1.4165
Asc	Φ_{ST} 0.0363* (0.0277) F_{ST} 0.0710** (0.0061)	–	Inf Inf	1.8475 2.8465	8.7769 8.5087	11.7403 7.7755
Bk	Φ_{ST} 0.0827* (0.0244) F_{ST} 0.0827* (0.0264)	Φ_{ST}−0.0087ns(0.7018) F_{ST}−0.0093ns (0.6295)	–	1.0699 1.5977	7.9071 4.5964	10.6343 4.5044
Pr	Φ_{ST} 0.6874* (0.0101) F_{ST} 0.6874** (0.0094)	Φ_{ST} 0.2130* (0.0284) F_{ST} 0.1494ns (0.0521)	Φ_{ST}0.3185* (0.0116) F_{ST} 0.2384* (0.0243)	–	39.8802 Inf	n.a. n.a.
ST	Φ_{ST}0.1291***(0.0000) F_{ST} 0.2937***(0.0001)	Φ_{ST} 0.0539* (0.0318) F_{ST} 0.0555* (0.0395)	Φ_{ST} 0.0595* (0.0270) F_{ST} 0.0981* (0.0139)	Φ_{ST} 0.0123ns (0.2743) F_{ST} −0.0211ns(0.5203)	–	n.a.
ST + Pr	Φ_{ST}0.0922***(0.0000) F_{ST} 0.2609***(0.0000)	Φ_{ST} 0.0409** (0.0185) F_{ST} 0.0604* (0.0195)	Φ_{ST} 0.0449* (0.0262) F_{ST} 0.0999** (0.0072)	n.a. n.a.	n.a. n.a.	–

Plo, Poilão; Asc, Ascension; Bk, Bioko; Pr, Principe; ST, São Tome. Significance values: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Below the diagonal are estimates of variation: Φ_{ST} (Excoffier et al. 1992) and F_{ST} (Weir and Cockerham 1984). Φ_{ST} was calculated using the Kimura 2-parameters model (assumes different substitution rates between transitions and transversions, and the ratio is computed from the data) with no gamma correction. *P*-values are in brackets. Above the diagonal are Nm estimates with and without correction for molecular distance (top and bottom line respectively). São Tome and Principe are shown separately and pooled. All comparisons between Comoros and Atlantic rookeries were significant [F_{ST} 0.3520–0.9784 ($P = 0.0000$)].

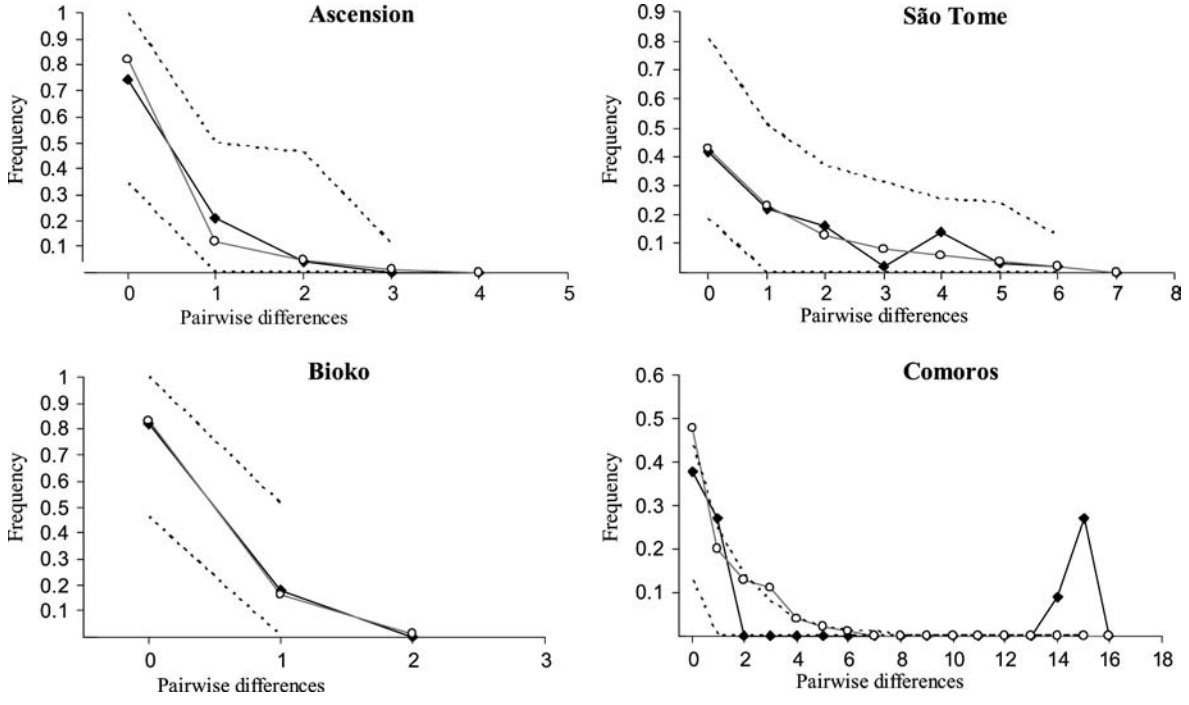

Figure 4. Mismatch distributions of four rookeries: Ascension ($\tau = 3$, $\Theta_0 = 0$, $\Theta_1 = 0.361$, SSD $P = 0.48$), São Tome ($\tau = 0$, $\Theta_0 = 1.475$, $\Theta_1 = 2.039$, SSD $P = 0.73$), Bioko ($\tau = 0.278$, $\Theta_0 = 0$, $\Theta_1 = 0.570$, SSD $P = 0.33$) and Comoros ($\tau = 6.234$, $\Theta_0 = 1.289$, $\Theta_1 = 1.289$, SSD $P = 0.23$). The solid black line with the ◆ marks is the observed distribution, the grey line with the ○ marks is the simulated distribution based on the sudden expansion model. Dashed lines represent the upper and lower confidence intervals at $\alpha = 0.05$ calculated using 100 replicates.

and Bioko had an N_0 of zero, while São Tome and Comoros had higher effective population sizes before expansion (581–2519 and 508–2201, respectively). The mismatch distributions of São Tome and Comoros both exhibited putative secondary expansion peaks, while Bioko and Ascension had steeply sloping concave curves, as expected in rapidly expanding populations.

Discussion

Genetic diversity

Ten out of the 14 haplotypes found among green turtle nesting females were previously undescribed. Seven of these were found in the Atlantic, while all three haplotypes described in Comoros were new. Ascension exhibited six haplotypes among 50 samples, while São Tome had seven among only 20 samples, but Poilão (n = 51) and Bioko (n = 50) were much less variable, with one and two haplotypes, respectively. We identified four previously undescribed haplotypes in São Tome and three in Ascension, most of which have not been found in any other nesting population, suggesting they may be unique to these rookeries. The fact that Bioko and Poilão exhibited small numbers of common haplotypes indicates they may have recently derived from a more diverse rookery. Sample sizes from Principe, Corisco and Comoros were relatively small and perhaps not adequate to represent the variation at these sites.

The highest values of haplotype and nucleotide diversity occurred in São Tome and Principe, followed by Ascension and Bioko. The smaller rookeries (each 75–100 nesting females per year) were substantially more diverse than the larger ones (3000–5000 and 400–600 nesting females per year in Ascension and Bioko, respectively). Interestingly, the more diverse rookeries were also those with smaller sample sizes. Nevertheless, each nesting population exhibited levels of haplotype diversity comparable to others of similar size in the Atlantic (Encalada et al. 1996), a pattern which has been reported before in green turtle rookeries (Allard et al. 1994; Lahanas et al. 1994). Several alternative explanations have been proposed: (1) Small populations exhibit higher levels of diversity as a result of the combined effects of immigration (such as due to imperfect homing behaviour) and the greater impact of recent admixture. Conversely, large populations with lower diversity may result from isolated founder events followed by rapid population increase; (2) Small populations with high diversity may be remnants of larger ancestral populations in the region (Allard et al. 1994; Lahanas et al. 1994). The mismatch distributions discussed below aided us in assessing these scenarios.

In the Indian Ocean, Comoros was significantly more diverse than the five rookeries sampled in the Atlantic and, despite small sample size (n = 10), its haplotype diversity (0.733) was remarkably similar to that calculated for the entire Indo-Pacific basin of 0.753 (Bowen et al. 1992).

The minimum spanning network consisted of two main haplogroups occurring in each ocean basin and separated by three equally parsimonious branches of 31 substitutions. The Comoros haplogroup was composed of three haplotypes (IND1–3) separated by one substitution. In addition, a partial sequence (NRC) found in the same rookery was distinguished by a minimum of 14 substitutions from the IND haplotypes. This rookery thus appeared to exhibit a deeper divergence within sequences than any of the Atlantic rookeries. Alignment with other published sequences from the Indo-Pacific shows substantial similarity between the IND haplotypes and haplotypes in the western Pacific (Dutton pers. comm.), and between NRC and haplotypes found in the south Pacific (Norman et al. 1994). The great geographic distance separating these similar haplotypes is interesting, but the lack of information on Indian Ocean phylogenies and our limited sample size, prevent us from speculating on overall Indo-Pacific phylogeography. The most parsimonious explanation for the presence of NRC is that it may represent a stray individual from a divergent population.

The Atlantic haplotypes grouped around a central and most common haplotype (CM8), likely to be the ancestral haplotype which gave rise to the others through single base pair mutations. Haplotype CM38, distinguished from its closest relative (CM8) by four substitutions, may be a representative of a divergent population that has become rare in recent history or a stray individual from an as yet unsampled population. The simplicity of the minimum spanning network and the small number of equally parsimonious solutions

allowed us to assess genetic relationships using molecular parsimony and allelic frequency criteria. It is likely that the rarer (and generally more recent) variants represent mutations of the most closely related common or ancestral haplotypes (Excoffier and Smouse 1994). Thus, with respect to the homoplasy observed in the Atlantic haplogroup, the least likely branching pattern probably involves a direct connection between CM46 and CM39 which, instead, are more likely to represent singleton branch tips than intermediates between the more common CM8 and CM6 haplotypes. The relatively small number of haplotypes and the weak separations between them imply a relatively recent origin for modern green turtle lineages in the Atlantic, a pattern noted previously for this species (Bowen et al. 1992) and for the leatherback sea turtle (*Dermochelys coriacea*) (Dutton et al. 1999).

Inter-oceanic population structure

Differentiation statistics indicated a deep evolutionary split and levels of gene flow close to zero between Comoros and Atlantic rookeries. Given that rates of mtDNA substitution can be used as an estimate of lineage divergence and colonisation time (Avise et al. 1992), we calculated that the Atlantic and Indo-Pacific ocean basins may have diverged approximately 3.6 million years ago (using our average mutation rate estimate of 0.01751 substitutions/site per Myrs). This observation is consistent with the closure of the Isthmus of Panama approximately 3 million years ago and is also comparable to the estimate by Bowen et al. (1992) using RFLP data of 1.5–3 million years. The temperate waters around the southern tip of Africa are generally considered a barrier to dispersal of green turtles (Hirth 1997). Nevertheless, zoogeographic studies have shown that the Cape of Good Hope is not an impenetrable barrier for many marine species, and is only approximately 96% effective as a barrier to dispersal of some tropical shore fishes (Briggs 1974).

The partial sequence NRC was separated from the IND haplotypes by more substitutions than the most divergent of the Atlantic haplotypes exhibited from one another over their entire sequence. Bowen et al. (1992) also showed deeper divergences within rookeries in the Indo-Pacific than within the Atlantic-Mediterranean using RFLP data, and the presence of divergent lineages within sea turtle rookeries in the Indian and Pacific Oceans has been noted elsewhere (Norman et al. 1994; FitzSimmons et al. 1997; Dutton in press). Bowen et al. (1994) found mitochondrial haplotypes belonging to different lineages in the same Florida nesting population of loggerheads (*Caretta caretta*). The olive ridley (*Lepidochelys olivacea*), another sea turtle with a tropical and subtropical distribution, is believed to have radiated to the Atlantic and East Pacific from an ancestral Indo-West Pacific stock (Bowen et al. 1998). Broderick and Moritz (1996) reported the co-existence of two divergent clades in single nesting stocks of hawksbills (*Eretmochelys imbricata*) in northeastern Australia. More comprehensive sampling is needed before we can speculate on explanations for the high divergence pattern observed within the Comoros rookery.

Population structure in the Atlantic

We found non-significant population differentiation between Ascension and Bioko and between Principe and São Tome. In addition, levels of gene flow were moderately high between Poilão and Ascension, Poilão and Bioko, Ascension and São Tome and between São Tome and Bioko, ranging around 5–13 individuals per generation. Gene flow higher than one migrant per generation was observed between all except one population pair (Principe and Poilão). Only 6–9% of the variation was partitioned among rookeries.

Observed population structure can result from processes over several spatial and temporal scales, including evolutionary and ecological time, geologic and climatic events, as well as behaviours such as philopatry and dispersal. Different levels of philopatry and nest-site fidelity have been reported in various regions and populations. Le Gall and Hughes (1987) recovered a nesting female tagged in Tromelin Island 2200 km away on another Indian Ocean nesting beach in Europa Island. Based on demographic and genetic studies, Norman et al. (1994) grouped nesting sites in Australia and Borneo separated by approximately 100–200 km into "rookery regions" containing single interbreeding populations. However, this region-specific philopatry has been questioned in other areas, where green turtles are known to exhibit high levels of beach-

specific philopatry (Allard et al. 1994; Peare and Parker 1996).

It is possible that our results do not necessarily indicate a breakdown in natal homing behaviour on an ecologically relevant timeframe, but rather, may be a reflection of recent colonisation (in the case of Bioko) and of occasional mistakes in natal homing (in the case of Principe, perhaps also biased by small sample size). Demographic considerations and geographic distance (2800 km) between Bioko and Ascension cast doubt on the fact that these two rookeries experience high levels of contemporary gene flow. On the other hand, proximity between the three rookeries in the Gulf of Guinea (Bioko, Principe and São Tome), separated from each other by a maximum of 400 km, may be more likely to result in occasional migrants preventing substantial differentiation at an evolutionary timescale.

Gene flow at evolutionary scales may be large enough to homogenise populations genetically but still not indicate strong demographic connection, particularly when population size is large (Avise 1995), as in the case of Ascension. On the other hand, Slatkin (1987) suggested that frequent extinctions and recolonisations can result in apparent gene flow between populations even in the absence of migration. Demographic turnover could lead to low inter-population differentiation if the population's persistence at a site is shorter than the time genetic drift takes to fix divergent alleles. Overall, frequent extinction and recolonisation events are likely to have shaped the distribution of green turtle rookeries, a dynamic process of natal homing behaviour and occasional homing mistakes leading to long-distance dispersal (Bowen et al. 1989, 1992; Meylan et al. 1990; Lahanas et al. 1994; Encalada et al. 1996).

Demographic history

Mismatch distributions were used to evaluate the demographic history of the nesting populations in an attempt to understand observed patterns of genetic diversity and distinguish between possible interpretations of shallow population structure: contemporary exchange of nesting females through imprecise homing (possibly facilitated by geographic proximity) versus common ancestry in relatively recent evolutionary time. While in stable populations the mismatch distribution is expected to be ragged and often multimodal, distributions observed in the rookeries analysed were mainly unimodal, thus indicating a population expansion following a bottleneck or founding event. This is consistent with the hypothesis that green turtle rookeries are formed by rookery turnover over evolutionary time as habitat availability varies with climatic fluctuations. Time since population expansion and coalescence time of haplotypes based on the mutation rate of sea turtle mtDNA were both found to be more recent than geological age of the island rookeries, which range from Bioko's one million years (Aka et al. 2001) to Principe's 31 million years (Lee et al. 1994). For instance, while the most divergent Atlantic haplotype (CM38) may have been derived approximately only 470,000 years ago, the rookery where it occurs (São Tome) is believed to be 13 million years old (Lee et al. 1994). Thus, more recent events are more likely to be responsible for the genetic structure observed today. In fact, population declines, extinction and recolonisation of rookeries, and range contraction and expansion due to climate and sea level changes, may have resulted in high levels of genetic drift, the disappearance of ancestral mtDNA lineages and lineage replacement of the precursors of modern haplotypes over evolutionary time.

Several studies have addressed the history of the Ascension Island population (Carr and Coleman 1974; Carr 1975; Bowen et al. 1989, 1992), suggesting an origin as recent as 10,000 years ago, in part based on the assumption that sea level changes during the last glaciation precluded nesting due to the steep cliffs above and below the water line (Bowen et al. 1992). However, our mismatch distribution indicated Ascension to be a rapidly expanding population, following a founding event ($N_0 = 0$) approximately 88,000 years ago, a more ancient origin than previously hypothesised. Interestingly, this time frame corresponds to a time of sea level rise between 130,000 and 70,000 years ago, when the sea reached levels similar to today's (Rohling et al. 1998).

The mismatch distribution of the São Tome population had a curve less smooth than that of other rookeries (Figure 4), with a minor peak suggesting a possible secondary expansion. São Tome was the only rookery in the Atlantic with a population size before expansion not estimated as zero, suggesting that it already hosted a nesting

population. We hypothesise that drop in sea level during Pleistocene glaciation events may have increased the amount of suitable nesting habitat available in São Tome (this tropical island is surrounded by shallow reef habitat, unusual along the west African coastline, and a gentle bathimetric gradient offshore), thus supporting a large ancestral population and possibly explaining the secondary expansion seen in the mismatch distribution. The predicted time of main expansion approximately 35,000 years ago corresponds to an estimated global sea level approximately 80 m below present levels (Rohling et al. 1998). Molecular evidence presented above (high diversity, unique and divergent haplotypes) also supports the hypothesis that the São Tome rookery may be the remnant of a large, diverse stock, and it is possible that nesting persisted in this ice age refugium as far back as the mid-Tertiary. Occasional immigration may have added further diversity or, vice versa, it may have served as a source for recolonisation of other rookeries in the region. On a contemporary timescale, São Tome may have been uninhabited until the arrival of the first Europeans in the 1470s, perhaps still supporting a large sea turtle population, which may have only recently declined to present levels as a result of overexploitation. While Principe's proximity to São Tome may suggest a similar demographic history, small sample size prevents us from speculating on its possible expansion patterns.

Bioko exhibited the most recent unimodal mismatch distribution, with expansion estimated approximately 8000 years ago, perhaps due to a founding event. Bioko's location on the continental shelf (32 km from the mainland, separated by waters less than 100 m deep) means that the 120 m drop in sea level at the end of the last ice age 20,000 years ago (Rohling et al. 1998) created a landbridge with the African continent. The timescale of expansion appears to be consistent with rookery colonisation following subsequent sea level rise and return to island status. Low genetic diversity, the presence of only two haplotypes, both common elsewhere and no rare or unique ones, also support the hypothesis that the Bioko nesting population derived from a recent founding event within the last 10,000 years. Bioko has probably been inhabited since the early Bantu expansions into Central Africa (approximately 3000 years ago) and turtles are likely to have been harvested at a low level for generations until commercial exploitation began in the 1900s (Butynski and Koster 1989).

Similarly to Bioko, Poilão's location on the continental shelf (50 km from the coast of Guinea Bissau) probably affected its island status during the most recent sea level changes. Thus, it may have a recent origin and demographic history comparable to Bioko. Colonisation resulting from imprecise natal homing, or perhaps from a southward range shift during climate change, may have led to the establishment of its single haplotype, followed by a rapid increase in population size, faster than the control region could accumulate mutations. An alternative explanation to lack of diversity in this population may be a recent bottleneck. However, the island's remoteness (25 km from the nearest inhabited island in the Bijagos Archipelago) and the apparent absence of contemporary exploitation pressure makes a recent decline in population numbers unlikely.

Finally, the predicted expansion of the Comoros rookery was found to be considerably older than the Atlantic rookeries ($t \approx 182{,}000$ years ago). The mismatch distribution showed two strong peaks (Figure 4) which, similarly to São Tome, might be attributed to secondary expansion (perhaps represented by the ''misdirected'' NRC haplotype). Volcanic activity during the recent formation of the Comoran archipelago 0.13–5.4 million years ago (Emerick and Duncan 1982) may have somehow affected demographic history. On the other hand, it is also possible that Comoros may be exhibiting features of a relatively stable population, a hypothesis consistent with the large estimate of t, deep sequence divergence, $N_1 \neq 0$ and $\theta_0 = \theta_1$. Perhaps Comoros has experienced only moderate population fluctuations over evolutionary time and relative stability may have allowed it to attain its currently large population size of approximately 5000 nesting females per season.

Conservation recommendations

A brief bottleneck, even if severe, does not necessarily lead to the loss of genetic variation within a population (Nei et al. 1975), as has also been shown for other sea turtle species (*Lepidochelys kempi*; Kichler et al. 1998). In addition, low mitochondrial and nuclear diversities are not always correlated, nor is mtDNA diversity clearly correlated

with expanding or declining populations (Moritz 1994b). Here, we have found that small populations (such as São Tome), which have presumably suffered declines in recent years, are still exhibiting relatively high mitochondrial variability, while others virtually none (Poilão). These results confirm the need for caution when making inferences on the relationship between mitochondrial and genomic diversity, and on the possible consequences of population decline on mtDNA diversity.

However, in the case of the migratory and elusive green turtle, defining the range for monitoring and conservation of populations at the level of nesting beaches and across regions should be an essential first step. Moreover, mixed groups with several contributing rookeries of origin are known to form in pelagic, developmental and feeding habitat, where they are often heavily exploited. Data presented here indicate the existence of two evolutionarily significant units (ESUs) as defined by Moritz (1994a), one in the Indian Ocean and one in the Atlantic Ocean, exhibiting high levels of genetic divergence and reciprocal monophyly at the mtDNA level. In order to evaluate patterns of genetic differentiation in the Atlantic which may help guide management decisions, we also applied the concept of management units (MUs). The MU definition is based on criteria such as recent divergence and difference in allele frequencies in subdivided populations, regardless of phylogenetic structuring (Moritz 1994b). We observed shallow population structure between several Atlantic nesting populations, probably as a result of common ancestry, recent colonisations, contemporary moderate levels of gene flow or demographic turnover. Nevertheless, according to MU criteria, Ascension and Bioko form a single MU, as well as São Tome and Principe, due to their homogeneous allele frequencies. Poilão represents a third, separate management unit since it revealed significant divergence in allele frequency when compared to the other rookeries. The genetic distinctness of these rookery units should be used to substantiate aggressive conservation measures.

Although Ascension and Bioko are grouped in a single MU, we argue for their importance as individual nesting populations due to their geographic location and distance. In this case, the diversity of the chosen molecular marker may be too low to assess population differentiation with the appropriate resolution. The presence of one very common haplotype and few rare ones may make it a comparatively insensitive marker. In addition, the allele frequency distribution of a matrilineal marker such as mtDNA may reflect historical connections and demographic events, which are not reflected in contemporary gene flow and dispersal (Avise et al. 2000).

Further, conclusions based on a single marker should be interpreted with caution; multiple genes, combined with nuclear markers, may provide substantially different estimates of migration and sex-biased gene flow, suggesting different MU designations. Incongruity between matrilineal and bi-parental genetic data has been observed on several occasions (see Moritz 1994b). In green turtles, microsatellites have been shown to be highly polymorphic and thus useful in fine-scale analyses (FitzSimmons et al. 1995). They can provide higher resolution than mtDNA to distinguish between populations on regional (FitzSimmons et al. 1997) and global scales (Karl et al. 1992; Roberts et al. 2004); the discrepancy between divergence detected through mitochondrial and nuclear markers was attributable to male-biased dispersal at feeding grounds, migration corridors and non-natal rookeries, or to overlapping breeding and migratory habitat.

Further research is needed to better describe conservation units in the region and their vulnerability to ongoing threats. In addition to genetic analysis of nuclear markers such as microsatellites, we recommend studies of direct measures of gene flow, including tagging programmes and satellite telemetry research throughout the region. The integration of observational and molecular data is essential to gaining an unbiased understanding of population structure and to designing an effective conservation strategy for these endangered populations.

Acknowledgements

Numerous people and organisations provided invaluable field assistance. Sampling in São Tome and Principe was possible thanks to J. Fretey (Museum National d'Histoire Naturelle-Paris, PROTOMAC- Protection Tortues Marines d'Af-

rique Centrale), A. Billes (PROTOMAC), C. Aveling, J. Rosseel, O. Neves and B. Giannuzzi-Savelli (ECOFAC- Conservation et Utilisation Rationnelle des Ecosystemes Forestiers d'Afrique Centrale, funded by the European Union). Sampling in Equatorial Guinea was supported by L. Arranz and J. Mba (ECOFAC), R. Castelo Alvarez (Asociación Amigos de Doñana, Bioko), J.E. Garcia, J.C. Serrano and Julian Nzi Mba (CUREF – Conservación y Utilización Racional de los Ecosistemas Forestales, funded by the European Union), D. Eparalele (Ureca), C. Epota Nassau (Corisco). Sampling on Ascension Island was assisted by A.C. Broderick, F. Glenn and G.C. Hays (University of Wales, Swansea, UK) in association with the Ascension Island Turtle Group (as part of projects funded by the Ascension Island Administrator, Darwin Initiative, Foreign and Commonwealth Office Environment Fund and NERC – Natural Environment Research Council, UK). Sampling in Guinea Bissau was supported by P. Catry, C. Barbosa, A. Almeida, B. Indjai, P.J. Perrida, J. da Silva (IUCN, Ministry of Agriculture and Rural Development, Centre for Applied Research on Fisheries, National Institute for Research, the People of Canhabaque). Sampling in Comoros was carried out by S. Ahamada (Marine Conservation Programme – AIDE). We gratefully acknowledge the following for granting permits: Direcçao Geral das Florestas e Caça (Guinea Bissau), Ministere de la Production et de l'Environnement (Comoros), Ministério da Agricultura e Desenvolvimento Rural (São Tome and Principe), Ministerio de Bosques y Medio Ambiente (Equatorial Guinea), Department of the Environment Transport and the Regions (UK). We are also grateful to G. Hewitt, G.C. Hays and J. Tomás for helpful suggestions, Paola Ciccarelli for her help with some analysis, and two anonymous reviewers for valuable comments on the manuscript. This work was supported by a grant from the Marie Curie Training and Mobility of Researchers Programme of the European Union to A.F., by European Union support to ECOFAC in São Tome and Principe (J.-F.D.), and by grants from the Convention on Migratory Species, the Fondation Internationale du Banc D'Arguin, NERC and People's Trust for Endangered Species to B.J.G.

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