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Conservation genetics of the east Pacific green turtle (*Chelonia mydas*) in Michoacan, Mexico

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Abstract

The main continental nesting rookeries of the east Pacific green turtle (EPGT), *Chelonia mydas*, on the Michoacan (Mexico) coast suffered drastic population declines following intense exploitation in the 1960s–1970s with annual abundance of nesting females plummeting from about 25,000 to an average of about 1400 between 1982 and 2001. Analyses of data from three nDNA microsatellite loci and 400 bp mtDNA control region sequences from a total of 123 nesting females sampled from four Michoacan rookeries found no evidence of population sub-structuring. The recent order of magnitude reduction in the population size shows no apparent impact on genetic diversity in either control region sequences (overall $h = 0.48$; $\pi = 0.0036$) or microsatellite loci (overall $N_a = 20.8$; $H_{exp} = 0.895$). Our estimates of annual effective female population size (N_{ef}; from $\theta = 2N_e\mu$) of $1.9–2.3 \times 10^3$, in spite of being an order of magnitude below historical records, appear to be sufficient to allow recovery of this population without significant loss of genetic diversity. These findings highlight the importance of continued conservation to reverse the decline of this population before it becomes vulnerable to genetic erosion.

Abbreviations: AB – Arenas Blancas; COL – Colola; EPGT – east Pacific green turtle; MAR – Maruata; mtDNA – mitochondrial DNA; nDNA – nuclear DNA; PN – Paso de Noria.

Introduction

The green turtle, *Chelonia mydas*, is distributed in tropical and sub-tropical waters and nests on beaches of five continents between latitudes 30°N and 30°S (Marquez, 1990; Hirth, 1997). Females of the species exhibit a strong tendency for natal homing and nest site fidelity (Meylan, Bowen and Avise, 1990; Bowen et al., 1995) that results in significant genetic differentiation among rookeries (Encalada et al., 1996). In the east Pacific the species has nesting sites from the Galapagos Islands (Ecuador) northward to Michoacan and the Revillagigedos Islands (Mexico), where it is known as the east Pacific green turtle (EPGT) or 'black turtle' (Pritchard and Mortimer, 1999). These individuals exhibit morphological characteristics that distinguish them from other *C. mydas* (e.g., adult carapace is much darker, domed, and with indented edges above the hind limbs; mature individuals are smaller and the breeding females lay fewer eggs; Hirth, 1997). These meristic features and multivariate morphological analyses have led some authors to consider the EPGT either as a full, separate species, *C. agassizii* (Bocourt, 1868; Pritchard, 1999), or a sub-species, *C. m. agassizii* (Marquez, 1990; Kamezaki and Matsui, 1995). However molecular genetic studies, which have demonstrated their utility in resolving major taxonomic and evolutionary controversies in marine turtles

(Bowen, Meylan and Avise, 1991; Bowen, Nelson and Avise, 1993), have instead indicated the EPGT to be simply a regional, melanistic population within the Pacific clade of *C. mydas* (Dutton et al., 1996; Karl and Bowen, 1999; Chassin-Noria, 2002).

The largest EPGT continental rookery (surpassed in size only in recent times by the population nesting in the Galapagos Islands; Hurtado, 1984; Zarate et al., in press) nests on a small number of beaches in the State of Michoacan (Mexico). However, these turtles have exhibited steep population declines due to commercial over-exploitation in the 1960s. The number of females nesting per year in the whole of Michoacan has collapsed from about 25,000 just prior to the 1970s (Cliffton, Cornejo and Felger, 1982), to an average of around 1400 between 1982 and 2001 (recalculated from Delgado and Alvarado, 2001); a period of roughly 30 years or about a single green turtle generation (Zug et al., 2002). By any standard, these trends indicate a critical condition which has lingered due to persistent take of animals at their foraging grounds in NW Mexico (Gardner and Nichols, 2001). The persistent endangered status of the Pacific Mexico nesting populations contrasts sharply with the status of *Chelonia* populations on the Atlantic coast of Mexico, where recent population increases (Arenas, Sarti and Ulloa, 2001) suggest positive results from similar multi-decade conservation efforts in that area.

Previous molecular genetic studies involving the EPGT have focused on phylogeography and taxonomy and were based on small sample sizes; a single individual from the Michoacan, Mexico rookery (Dutton et al., 1996), and five individuals from Galapagos, Ecuador (Karl and Bowen, 1999). Here we present results from extensive sampling of the four EPGT rookeries that remain along discontinuous beaches in Michoacan (Mexico) and analyze the distribution of genetic variation within two types of molecular markers with complementary features. The mitochondrial DNA (mtDNA) control region, being maternally inherited, is characterized by a rapid rate of evolution and a higher proportion of genetic variance among populations than for bi-parentally inherited markers (Cann, Stoneking and Wilson, 1987). This molecular tool has been extensively used to investigate molecular systematics and population genetic differentiation in marine turtles (Moritz, Dowling and Brown, 1987; Norman, Moritz and Limpus, 1994; Avise, 2000), and its broad application in other sea turtle species (e.g., Norman, Moritz and Limpus, 1994; Encalada et al., 1996; Bowen et al., 1998; Dutton et al., 1999) allows for comparisons of genetic attributes with previous studies. Microsatellites, on the other hand, are a highly polymorphic class of nuclear DNA (nDNA) markers which have been applied in sea turtles to study identity and paternity (Dutton, 1996a; FitzSimmons, 1998; Crim et al., 2002), regional geographic structuring and male-biased gene flow (FitzSimmons et al., 1997).

This study extend the geographic, numeric and genetic coverage of surveys on the EPGT nesting in Michoacan, to (1) evaluate levels of genetic diversity and, by comparing with analogous studies in other marine turtle species, detect potential evidence that population declines have caused an erosion of genetic variability in the EPGT; (2) study the extent of genetic structuring among rookeries; (3) use the genetic data to estimate historical effective population sizes (N_e) in order to evaluate the extent to which population crashes have affected the abundances of breeding populations.

Materials and methods

Sampling and laboratory procedures

A total of 123 samples were obtained from females nesting at four beaches in Michoacan, Mexico (Figure 1) – Colola (COL, $n = 70$), Maruata (MAR, $n = 19$), Paso de Noria (PN, $n = 15$), and Arenas Blancas (AB, $n = 19$) – during two breeding seasons (September 1996 – January 1997 and September 1997 – January 1998). Differences in sample sizes are due to limitations in availability, particularly from PN and AB, where fewer than 100 females nest every season. When nesting females were found, blood samples (3 ml) were extracted from the dorsal cervical sinus immediately after egg laying (Owens and Ruiz, 1980). After sampling, a Monel tag was applied to the right front flipper for identification to avoid duplicate sampling. Blood was preserved in 1–5 ml lysis buffer (100 mM Tris–HCl pH 8; 100 mM EDTA pH 8; 10 mM NaCl; 1.0% (w/v) SDS; Dutton, 1996b). When the nesting female was not observed and only its nest was available, a single dead hatchling was collected from each nest. Muscle biopsies of dead hatchlings (~3 mm^3) were suspended in 'SED' buffer (20% DMSO, 250 mM EDTA, saturated NaCl, pH 7.5–8). All nest samples were taken within an 11-day period to avoid resampling females that commonly nest 2–4 times per season in cycles of approximately 14-days (Hirth, 1997).

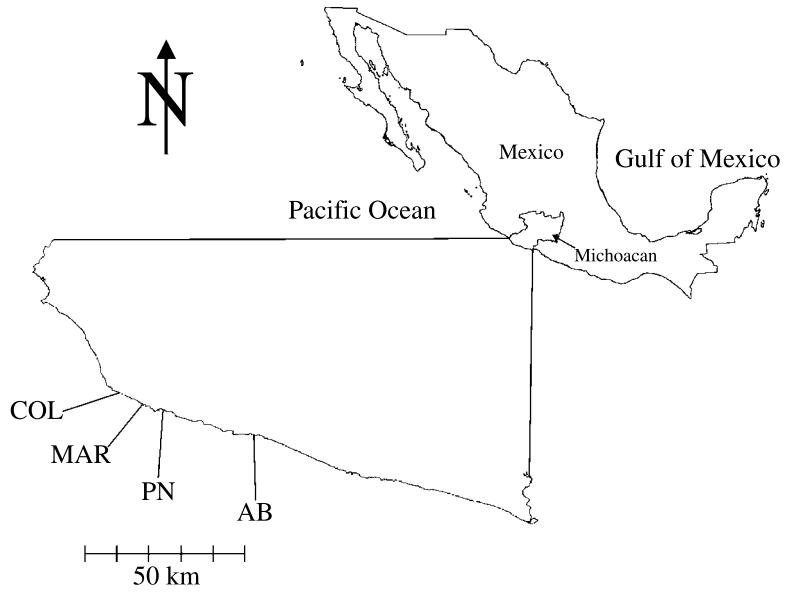

Figure 1. Location of EPGT rookeries sampled in Michoacan State (Mexico). COL remains the single most abundant continental EPGT rookery in the east Pacific.

We extracted genomic DNA using the proteinase K digestion protocol of Maniatis, Fritsch and Sambrook (1982) with the modifications described by FitzSimmons (1997). Approximately 20 µl of blood in lysis buffer were incubated in 300 µl buffer (10 mM Tris; 1 mM EDTA; 10 mM NaCl and 0.1% SDS), with proteinase K (final concentration of 1 mg/ml) for 1–3 h at 60°C with occasional tube inversions. DNA was precipitated with ethanol, and resuspended in 50–100 µl H_2O.

For the mtDNA control region we amplified 2 µl of template DNA in 25 µl reaction volumes (containing 200 µM dNTP's, 1.5 mM MgCl, 1 µM each primer, 1.5 Units *Taq* polymerase) with 30 PCR cycles (Saiki et al., 1988), using a Perkin-Elmer Inc. thermal cycler with the following protocol: DNA denaturing at 94°C for 1 min, primer annealing at 57°C for 1 min, and primer extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. LTCM1 and HDCM1 primers (Allard et al., 1994) were used, which amplified a portion of about 550 bp from the 5′ end of the region, although sequences were truncated at 400 bp for subsequent analyses to homogenize sequence lengths. Sequences were obtained using an Applied Biosystems Inc. (ABI model 377) automated sequencer, with all variable positions confirmed by comparing sequences from both strands.

PCR conditions for microsatellite amplifications were the same as for the mtDNA control region, except that 10-s steps were used in 35 amplification cycles. Cm3, Cm72, and Cc7 primers (FitzSimmons, 1997) were used for nDNA microsatellite amplification. The PCR products were run on 4% denaturing polyacrylamide electrophoresis gel (44 cm × 33 cm × 0.04 cm plates) prepared as described by Caetano-Anolles and Gresshoff (1994). Electrophoresis was performed at ~60°C until the bromophenol blue dye marker reached the leading edge of the plates. Electromorphs were silver stained as described by Caetano-Anolles and Gresshoff (1994). A 123 bp marker was included in all gels as size marker in both margins. Allele sizes were determined using Labimage 2.02 (Labsoft Diagnostics AG) software.

Data analysis

Estimates of haplotype nucleotide compositions and population genetic variation (haplotype, h, and nucleotide, π, diversities) were derived from aligned (Clustal V; Higgins, Bleasby and Fuchs, 1991) mtDNA haplotypes, using Arlequin 2.0 (Schneider, Roessli and Excoffier, 2000). This program was also used to estimate gene flow values (as N_m), F_{st} values, to perform analyses of molecular variance (AMOVA) and to derive minimum spanning networks.

The mtDNA sequence divergence between populations or haplotypes was quantified using total (d_{TOT}) and net (or 'corrected', d_{NET}) average number of pair-

Table 1. mtDNA control region haplotype frequencies at four Michoacan EPGT rookeries. (GenBank accession numbers AY382323-AY382327, with position 1 corresponding to position 15630 of *C. mydas* complete mtDNA sequence of GenBank accession number 15630)

Rookery	*n*	Haplotype frequency				
		CMP4	CMP5	CMP7	CMP8	CMP12
Colola	70	0.69	0.29		0.03	
Maruata	19	0.53	0.42	0.05		
Paso de Noria	15	0.87	0.13			
Arenas Blancas	19	0.58	0.26			0.16
Overall	123	0.67	0.28	0.01	0.02	0.02

wise differences (Nei, 1987, Eqs. 10.20 and 10.21, respectively). The latter corrects for intrapopulational variation. Published mtDNA control region sequences for Atlantic *C. mydas* (Encalada et al., 1996) and Pacific *C. mydas* (Norman, Moritz and Limpus, 1994) were compared in order to estimate genetic divergence between Pacific and Atlantic *Chelonia*. Since data are not available for all populations, haplotype frequencies were input into the analysis software as $1/n$, where $n =$ number of haplotypes, since all haplotypes are different and none are shared between groups of populations evaluated (following Nei, 1987, p. 276). The Tamura (1992) method for multiple mutations correction was used since this and previous results with *Chelonia* (e.g., Encalada et al., 1996) indicate very high transition:transversion bias ($\gg 8$) and unbalanced base frequency composition with $A + T \gg G + C$. All of the above computations were calculated using Arlequin 2.0 (Schneider, Roessli and Excoffier, 2000).

Long-term effective population size of females (N_{ef}) was calculated following the approach described by Cummings and Clegg (1998), from estimates of nucleotide polymorphism, π, which is equivalent to $2N_e\mu$ in haploid systems. The θ statistic was estimated using Arlequin 2.0 (Schneider, Roessli and Excoffier, 2000) on the basis of the number of segregating sites in a sequence (θ_S) and the average number of pairwise differences between sequences, π (θ_π). To obtain N_{ef}, a corresponding mutation rate per lineage (μ) for control region sequences was estimated in two steps: (1) using Arlequin to estimate d_{NET} between published sequences from Atlantic (Abreu-Grobois et al., 1996; Encalada et al., 1996) versus Pacific *Chelonia* populations (Norman, Moritz and Limpus, 1994; Abreu-Grobois et al., 1996), and (2) calibrating this estimate with the rise of the Panama Isthmus, 3.5 Mya (Coates et al., 1992). In the calculations, time was converted to *Chelonia* generations by assuming a mean of 40 years/generation (an approximation based on an estimate of 30 years for time to maturity by Zug et al. (2002) plus half reproductive longevity of about 20 years, using Pianka (2000, Eq. p. 141)). In order to compare to recent population values, the N_{ef} was expressed in terms of nesting females/year by assuming that, on an average, *Chelonia* females nest every 3 years (average from data in Hirth, 1997).

Divergence in microsatellite allele frequencies among rookeries was tested by comparing resulting chi-squared values with 10,000 randomized data sets generated by REAP (McElroy et al., 1992), a test performed to compare our results with those of previously published work on *Chelonia* populations (Karl, Bowen and Avise, 1992; FitzSimmons et al., 1997). Deviation from Hardy–Weinberg equilibria were tested with Genepop version 3.3 (Raymond and Russet, 1995), F_{st} estimations were obtained with Arlequin

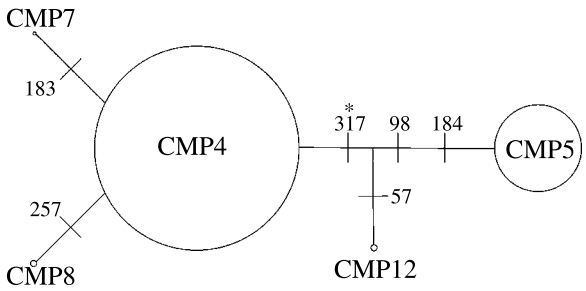

Figure 2. Network describing evolutionary relationships among the Michoacan EPGT mtDNA control region haplotypes in Table 1. An asterisk indicates the single parsimony-informative site; the circles' sizes are proportional to haplotype frequencies in the whole of Michoacan. The numbers represent the polymorphic positions in our 400 bp haplotypes, where position 1 corresponds to position 15,630 of the *C. mydas* mitochondrial genome in GenBank AB012104.

Table 2. mtDNA control region sequence diversity in Michoacan EPGT, as measured by haplotype diversity (h) and nucleotide diversity (π). n denotes sample size, values in parenthesis are standard errors. As no significant genetic differentiation was detected between seasons for any of the rookeries (results not shown), data for the two sampled seasons were grouped for each rookery. Values for other species are included for comparison

Rookery	n	h	π	Reference
Michoacan (grouped results)	123	0.48 (0.04)	0.0036 (0.0024)	This paper
Colola	70	0.45 (0.05)	0.0033 (0.0023)	This paper
Maruata	19	0.57 (0.06)	0.0041 (0.0028)	This paper
Paso de Noria	15	0.25 (0.13)	0.0018 (0.0016)	This paper
Arenas Blancas	19	0.60 (0.09)	0.0052 (0.0034)	This paper
Other species				
C. mydas (Atlantic)	8–15	0.200–0.618	0.004–0.0053	Lahanas et al. (1994)
C. caretta (Atlantic and Mediterranean)	11–105	0.00–0.67	0.000–0.0294	Encalada et al. (1998)
Eretmochelys imbricata (Atlantic)	14–15	0.125–0.782	0.0012–0.0060	Bass et al. (1996)
Lepidochelys olivacea (global)	80	0.00–0.81	0.000–0.0207	Bowen et al. (1998)
L. kempii	9	0.69	0.0033	Bowen et al. (1998)

2.0 (Schneider, Roessli and Excoffier, 2000), and R_{st} values with RSTcalc 2.2 (Goodman, 1997).

Results

As is common in other marine turtle species (Karam, 1997), nucleotide composition of EPGT sequences was heavily biased towards A/T bases (68% relative composition). A total of five 400 bp haplotypes were detected among the 123 samples amplified for the mtDNA control region (Table 1) which resulted from six polymorphic positions, all transitions, of which only one (position 317) was parsimony-informative. The observed haplotypes are closely related, differing in at most four positions (Figure 2). Haplotypes CMP4 and CMP5 were the most common in all four rookeries, with frequencies >53 and >13%, respectively and, in combination, they represent >84% of the individual population's total haplotype complement. The remaining three, CMP7, CMP8 and CMP12, were rare (<20%) and each was only found in single rookeries (Table 1). None of these haplotypes has ever been reported from any *Chelonia* population, although the most important rookeries have already been surveyed (Norman, Moritz and Limpus, 1994; Encalada et al., 1996; P. Dutton et al., in preparation).

Haplotype diversity (h) varied from 0.25 (PN) to 0.60 (AB), similar to estimates from other marine turtle species (Table 2). Nucleotide diversity (π) varied from 0.0018 (PN) to 0.0052 (AB), within the ranges found in other species (Table 2). Global values (obtained by grouping all results) were $h = 0.48$ and $\pi = 0.0036$.

The three microsatellite loci analyzed were highly variable. In order of variability, Cm72 had 53 alleles, Cc7 had 33, and Cm3 had 27 alleles, and intermediate-sized alleles were found at highest frequencies (see Figure 3). Heterozygosity and number of alleles per locus were very high at all rookeries (mean H_{obs} (overall loci) > 0.95, and $H_{exp} > 0.87$; the number of alleles varied from 11 to 46) (Table 3). No deviations from Hardy–Weinberg equilibrium were found after adjusting for multiple tests ($n = 12$) according to the Bonferroni correction (Rice, 1989). Allele frequency differences among EPGT rookeries were not significant for any of the loci assayed (Cm3: $\chi^2 = 93.29$, $P = 0.133$; Cm72: $\chi^2 = 173.25$, $P = 0.146$; Cc7: $\chi^2 = 100.71$, $P = 0.349$).

The AMOVA results for both the mtDNA sequence and microsatellite data indicate that, respectively, 98.8 and 99.7% of genetic variability resides within rookeries (data not shown). Nonetheless, there is no indication of inbreeding within localities (multilocus $F_{IS} < 0.09$; Table 3).

Estimates of genetic differentiation between the four populations were very low, both from mtDNA sequences and microsatellite data. For mtDNA, F_{st} ranged from 0.004 to 0.123. The two methods used with the microsatellite data produced similar results: F_{st} ranged from 0.0012 to 0.0060, and R_{st} values

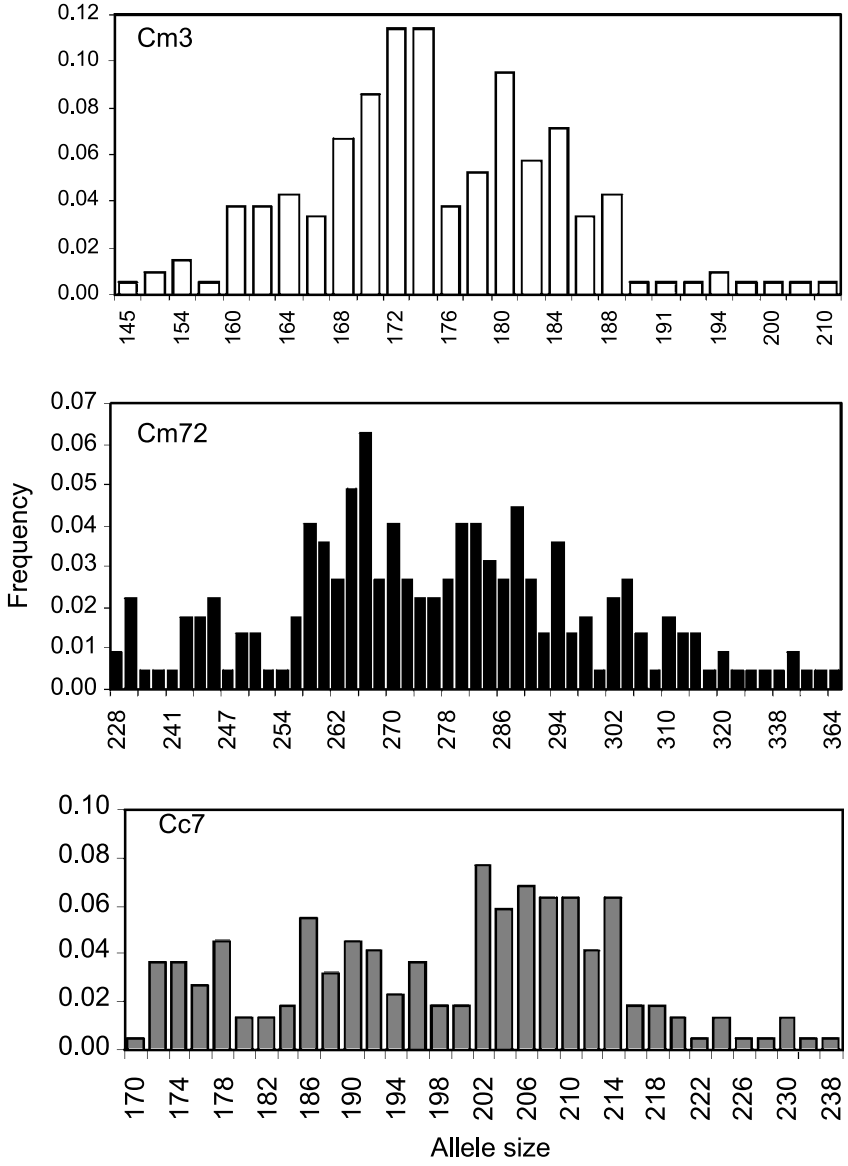

Figure 3. Allele frequency distribution for three microsatellite loci (Cm3, Cm72, and Cc7) in Michoacan EPGT rookeries. Allele frequencies were grouped for all rookeries as no statistically significant heterogeneity was detected (see text).

from negative values (i.e., no genetic differentiation) to 0.024 (Table 4).

The estimated numbers of migrants per generation among localities (in terms of N_m) were all >38 from the microsatellite data. Estimates of N_m from mtDNA data, with the exception of a low value for the MAR versus PN analysis ($N_m = 3.6$) had values >20. Considering the above results and that migration rates (N_m) of more than 1–10 allow sufficient gene flow to curtail interpopulational differentiation (Mills and Allendorf, 1996), our results support a view that all rookeries in Michoacan behave as a single population. Thus the populations were pooled for subsequent analyses.

Estimates of effective population size

If a specific mutation rate, μ, is assumed, then estimates of θ from grouped rookery results can be used to estimate N_e, the effective population size, for the

Table 3. Summary of genetic variation at three microsatellite loci for EPGT rookeries in Michoacan (see text for site codes)

Locus	Rookery				Mean (N_A/locus)
	COL	MAR	PN	AB	
Cm3					
n	62	14	10	17	
N_A	23	13	11	14	15.3
H_{exp}	0.98	1.00	0.80	0.88	
H_{obs}	0.94	0.94	0.97	0.94	
P	0.16	0.83	0.13	0.12	
Cm72					
n	64	15	15	19	
N_A	46	19	19	25	27.8
H_{exp}	0.84	0.80	1.00	1.00	
H_{obs}	0.98	0.97	0.96	0.97	
P	0.00	0.07	0.53	1.00	
Cc7					
n	62	16	15	18	
N_A	33	16	13	17	19.8
H_{exp}	0.90	1.00	0.80	0.72	
H_{obs}	0.96	0.95	0.95	0.95	
P	0.04	0.81	0.19	0.02	
Mean N_A/ location	34.0	16.0	14.3	18.7	20.8
Mean H_{exp}	0.91	0.93	0.87	0.87	
Mean H_{obs}	0.96	0.95	0.96	0.96	
Multilocus F_{IS}	0.06	0.06	0.07	0.09	

n = number of individuals sampled; N_A = number of alleles; H_{exp} = unbiased expected heterozygosity (Nei, 1978); H_{obs} = observed heterozygosity; and P = probability for deviation from Hardy–Weinberg equilibrium. No significant deviations from H–W equilibrium were found after sequential Bonferroni correction (Rice, 1989).

Table 4. Measures of genetic sub-division among EPGT rookeries in Michoacan. (A) Pair-wise F_{st} estimates from mtDNA control region sequence data. (B) Microsatellite pair-wise testing for all loci R_{st} values, above the diagonal, and F_{st} values, below the diagonal. None of the comparisons exhibited significant deviations from 0 at $\alpha = 0.05$ in permutation testing

(A)

	Colola	Maruata	Paso de Noria
Maruata	0.004		
Paso de Noria	0.024	0.123	
Arenas Blancas	0.020	0.029	0.023

(B)

	Colola	Maruata	Paso de Noria	Arenas Blancas
Colola		0.024	0.004	0.002
Maruata	0.006		−0.036	0.026
Paso de Noria	0.005	0.002		0.002
Arenas Blancas	0.002	0.001	0.002	

whole of Michoacan, by using a transformation of the original Watterson (1975) equation $N_e = \theta/2\mu$ (for haploid systems such as mtDNA). As female lineages are assayed when working with mtDNA, we can only calculate effective population sizes of females (N_{ef}). We estimated μ from net genetic distances between published *Chelonia* haplotypes from Pacific (Norman, Moritz and Limpus, 1994) and Atlantic populations (Encalada et al., 1996), calculated using the Tamura (1992) model and Nei (1987) formula 10.21. The haplotypes frequencies were entered as 1/total number of haplotypes for computations as there is no haplotypes overlap among the two groups of populations (following Nei, 1987, p. 276), assuming that these two lineages were split by the closing of the Isthmus of Panama (Coates et al., 1992). The resulting mutation rate that we calculated for *Chelonia* (0.62% per MY per lineage) is similar to the values used by Lahanas et al. (1994) of 1.76% per MY per pair of lineages and Encalada et al. (1996) of 1.2–2.4% per MY per pair of lineages, and 1.7–2.2% per MY per pair lineages (Dutton et al., 1999) (equivalent to 0.88%, 0.6–1.2%, and 0.88–1.1 per MY per lineage, respectively); although we have employed more extensive sequence comparisons. Assuming a generation time of 40 years, based on the estimate in Zug et al. (2002) of 30 years to sexual maturity and adding half reproductive longevity of about 20, following Pianka (2000) equations, our results lead to an estimated generational mutation rate per lineage of 2.48×10^{-7} mutations per nucleotide site.

Estimates of θ per site combining the five Michoacan EPGT haplotypes, ranged from 0.0028 (based on number of segregating sites, S_n, from Watterson, 1975) to 0.0034 (based on mean number of pairwise differences, π, from Tajima, 1983). These θ values were used to approximate N_{ef} for EPGT females nesting in the whole of Michoacan. From estimates of mutation rate per site per generation of 2.48×10^{-07}, N_{ef} was calculated at 5610 based on S_n and of 6800 based on π, equivalent to 1860 and 2260 females nesting per season, respectively if one assumes EPGT mature females effectively only nest on average every 3 years (Hirth, 1997).

Discussion

Evaluations of genetic diversity are of use to wildlife managers, particularly in cases, such as the EPGT, where there has been a sudden, drastic population decline that may have led to loss of genetic variability. Loss of genetic variability is believed to diminish the ability of populations to adapt or respond to environmental change (Frankel and Soule, 1981; Lacy, 1987). Small populations are most vulnerable to effects of loss of variability as rates are dependent on the effective number of breeders in a population (N_e) and time (Crow and Kimura, 1970). Thus, managers place priority on identifying populations that are both small in terms of N_e and that have recently lost genetic variability, as these may be more susceptible to demographic and genetic stochasticity (Mills and Smouse, 1994). The Michoacan EPGT has been under these considerations due to fears of potential genetic erosion provoked by the high exploitation rates to which they have been subjected.

The extent of genetic diversity at the mtDNA control region in the Michoacan EPGT rookeries is reflected in a total of five closely related haplotypes, with a typical 'star shaped' phylogeny having a central haplotype found at the highest frequency (CMP4) that also represents the most probable ancestral sequences from which remaining sequences, particularly haplotypes CMP7, CMP8, and CMP12, would have recently derived (Figure 2, Table 1). This would probably have occurred within this region (i.e., in relative isolation) since (a) at most four base substitutions (all transitions) exist among Michoacan haplotypes, and (b) the two most common haplotypes (CMP4 and CMP5) are only found in EPGT populations, and all low frequency haplotypes (CMP7, CMP8, and CMP12) have been found exclusively in Michoacan rookeries (P. Dutton et al., in preparation). The assumption of local evolution of the genetic diversity at this locus is critical for the estimation of effective population size in this population (see below).

Nucleotide and gene diversity estimates for the Michoacan rookeries, which are within the range reported for other marine turtles (Lahanas et al., 1994; Bass et al., 1996; Bowen et al., 1998; Encalada et al., 1998), may also provide insight into the demographic history of the population (Grant and Bowen, 1998; Avise, 2002). While data from a broad suite of populations are necessary to perform this analysis in a thorough fashion, available information from green turtles provides enough background to derive preliminary conclusions for important demographic aspects of the Michoacan population.

First, the haplotype diversity value ($h = 0.48$) is within the high end of the range found in other marine turtles (Table 2) suggesting that this population has not undergone a genetic bottleneck (see also discussion in section on effective population size, below). The history of intense human exploitation of Michocan EPGT does not appear to have reduced the number of nesting females below levels known to be critical in provoking negative genetic impacts due to bottleneck effects (i.e., population size <20 according to Glenn, Stephan and Braun, 1999; Whitehouse and Harley, 2001).

Second, high genetic diversities, while positively correlated with population size in theory (Soule and Khom, 1989; Frankham, 1996), can also be the result of multiple colonization events, or of immigration by differentiated populations. A comparison of our genetic diversity estimates with those from previous studies allows this to be explored. For example, immigration or genetic admixture, in spite of a general phylopatric behavior of marine turtles, have been used to explain the relatively high values of both haplotype and nucleotide diversity found in some relatively small green turtle rookeries: Polynesia ($h = 0.44$, $\pi = 0.0027$, Number of females population = few hundred; Bowen et al., 1992); Aves Island, Venezuela ($h = 0.25$, $\pi = 0.0053$; Number of females population = 300–500, Lahanas et al., 1994); and Quintana Roo, Mexico ($h = 0.82$, $\pi = 0.0057$; Number of females population = 100–400, Encalada et al., 1996). In all these cases, the high π values respect to the other beaches surveyed on each work, have been interpreted as reflecting historical introgressions by individuals with divergent haplotypes. In the case of the Michoacan rookeries in our study, the relatively low π value coupled with relatively high haplotype diversity and the low frequency of three of the five haplotypes found suggest that the three lowest frequency haplotypes were originated from a localized evolutionary process rather than immigration.

Another argument against the existence of a bottleneck is the relatively high genetic diversity in the Michoacan EPGT reflected by the high number of alleles for microstellite loci and with broad frequency distributions (Figure 3). This is particularly the case for Cm72 (with a total of 53 alleles), a locus that has been shown to accumulate site-specific mutations at very high rates (FitzSimmons, 1998).

The expected heterozygosities estimated for this locus ($H_{exp} = 0.80–1.00$) in Michoacan are comparable to those obtained for Australian green turtle populations ($H_{exp} = 0.87–0.96$) (FitzSimmons et al., 1997). These populations have average nesting numbers that range between thousands to tens of thousands of individuals (Limpus, Miller and Parmenter, 1993), and is considered the biggest green turtle nesting aggregation in the world. This comparison suggests that genetic erosion has not occurred in Michoacan green turtles, despite of the severe population decline.

Genetic structure

Genetic research has revealed extensive population sub-structuring within the geographic ranges of all sea turtle species studied to date (Bowen et al., 1992; Bowen et al., 1994; Encalada et al., 1996; FitzSimmons et al., 1997; Bowen et al., 1998; Dutton et al., 1999), confirming the theory of natal homing by females (Meylan, Bowen and Avise, 1990; Allard et al., 1994) and to some extent also for males (FitzSimmons et al., 1997). However, these studies have been global in scale (Bowen et al., 1992) or covered extensive geographic ranges (>1500 km) (e.g., Encalada et al., 1996; FitzSimmons et al., 1997). Our failure to detect genetic sub-structure of female lineages on a finer scale (45 km between the extreme rookeries, COL and AB) suggests that the precision of natal homing operates over larger geographic scales, at least on evolutionary time scales that would be reflected at the level of the genetic markers we used, and that the Michoacan rookeries comprise one genetic stock for management purposes. These results should be interpreted with caution and in conjunction with other lines of evidence, since genetic results may fail to detect more subtle demographic isolation between populations (Taylor and Dizon, 1996). However, the available tagging studies demonstrate that some females nest both in COL and MAR (J. Alvarado, personal communication). This would be compatible with our genetic results and would further corroborate the notion of Michoacan rookeries comprising a cohesive management unit.

Effective population size

The effective population size (N_e, harmonic mean size of the breeding population over a historic timescale) is one of the most valuable parameters for management. Yet, its estimation presents practical obstacles when attempting to determine it using a purely demographic approach as it is almost impossible to identify the numbers of breeders and the value is most sensitive to differential breeding variances either between sexes or across breeding seasons. It can, however, be approximated using genetic diversity information (e.g., Cummings and Clegg (1998) for wild barley; Lahanas et al. (1994) for *Chelonia* populations; Dutton et al. (1999) for *Dermochelys*).

Our estimates of between 1900 and 2300 breeding females per season (N_{ef}) are about 1/10 the population size reported in the late 1960s of 25,000 (Cliffton, Cornejo and Felger, 1982) but the same order of magnitude of current census sizes, estimated to be around 1400 females per year (Delgado and Alvarado, 2001) calculated between 1997 and 2001 for Colola and assuming that nesting abundance on this beach represents a 60% of whole of Michoacan (Delgado and Alvarado, 2001). While caution should be exerted when reaching conclusions based on genetic diversity-based N_e calculations which rely on a number of assumptions, experience with other species do provide useful insights. For example, even in shellfish and widely distributed marine fish with extremely high fecundities, N_e is estimated in this manner are typically an order of magnitude lower than present day population sizes (Grant and Bowen, 1998). The proportion of N_e values from Michoacan relative to past population sizes is very similar to the results found in *Dermochelys* (Dutton et al., 1999) and may be a general pattern for slow-growing, late maturing species. Nevertheless, although further population declines in the Michoacan green turtle, if unchecked across several generations, could lead to critically small effective population sizes (Frankel and Soule, 1981) due to genetic effects of inbreeding (Hartl and Clark, 1989). The inferred N_e is large enough to guarantee the future of this population if we consider that the values suggested to avoid negative impacts from genetic drift and inbreeding are, respectively, two and one orders of magnitude smaller (Franklin, 1980) than those we report here for the Michoacan population.

Conservation implications

The absence of detectable genetic structure in the Michoacan EPGT should not preclude continuation of conservation efforts encompassing all rookeries in

this area, and those further south in the country. Our genetic results would indeed suggest significant inter-rookery genetic exchange over evolutionary timescales ($N_m > 38$ from the microsatellite data and >3.5 from mtDNA data), that would provide the potential for restocking and sustaining individual rookeries in the region. However, past experience of extirpation of many historic EPGT rookeries along the Pacific coast of Mexico (e.g., those in Jalisco and Oaxaca States, where green turtles were known to have been abundant in the past) suggests that the speed with which human interference and human-induced environmental change manifest themselves can be overriding.

Ongoing genetic studies (Dutton et al., in preparation) on a broader geographic scale provide further insight into patterns and magnitude of inter-regional genetic exchange. An expanded, region-wide tagging program to complement and augment genetic results, in order to better understand long-term abundance trends, as well as the extent of short-term inter-rookery movements. This information will be needed to guide robust management strategies that may guarantee the long-term survival of the EPGT.

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