

# POPULATION GENETIC STRUCTURE OF GREATER CARIBBEAN GREEN TURTLES (*Chelonia mydas*) BASED ON MITOCHONDRIAL DNA SEQUENCES, WITH AN EMPHASIS ON ROOKERIES FROM SOUTHWESTERN CUBA.

Ariel Ruiz-Urquiola <sup>1</sup>, Frander B. Riverón-Giró <sup>1</sup>, Emir Pérez-Bermúdez <sup>1</sup>, Francisco A. Abreu-Grobois <sup>2</sup>, Maribel González-Pumariega <sup>1</sup>, Benjamin L. James-Petric <sup>3</sup>, Rogelio Díaz-Fernández <sup>1</sup>, José M. Álvarez- Castro <sup>4</sup>, Murier Jager <sup>5</sup>, Julia Azanza-Ricardo <sup>1</sup> & Georgina Espinosa-López <sup>1\*</sup>.

- (1) Centro de Investigaciones Marinas, Universidad de La Habana, Calle 16 No. 114, CP 11300, playa, Ciudad Habana, Cuba  
(2) Unidad Académica Mazatlán, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Apdo. Postal 811, Mazatlán, Sinaloa, CP 82000, México.  
(3) Faculty of Environ. Studies, York University, HNES 109, York University, 4700 Keele Street, Toronto, Ontario, M3J 1P3, Canada.  
(4) Linnaeus Centre for Bioinformatics, Uppsala University. BMC, Box 598, SE-751 24, Uppsala, Sweden.  
(5) Univ. Pierre et Marie Curie-Paris 6, UMR 7138 CNRS UPMC MNHN ENS IRD, Case 05, 9 quai St Bernard, 75005 Paris, France.  
(\*) Corresponding author: Email: [georgina@fbio.uh.cu](mailto:georgina@fbio.uh.cu); [georgina@cim.uh.cu](mailto:georgina@cim.uh.cu)

## ABSTRACT

Effective conservation management of sea turtles depends on understanding the demographic interconnections between populations. Beginning with the sequencing of 490 bp from the mitochondrial DNA control region, we characterized genetic variation within and between two green turtle (*Chelonia mydas*) rookeries in southwestern Cuba [N=28] and assessed these data in a regional context to understand the contribution of each rookery to the genetic structure of the species. In the Cuban rookeries, haplotypes belong to the same lineage (II) and 71% of them are endemic but in low frequency. There was no significant genetic structure among Cuban rookeries. However, when compared with the rest of the Greater Caribbean rookeries, some genetic structuring was revealed (AMOVA), with higher variation within than between rookeries. Genetic differentiation ( $F_{ST}$ ) was positively correlated with geographical distances among rookeries. In the Cuban population, a pattern of sudden expansion was supported by the observed mismatch distribution. However, Fu's test failed to reject the standard neutral model, probably as a consequence of recent migrations. Using a nested clade phylogeographic analysis, restricted genetic flow and isolation by distance for the clade containing most of the Cuban haplotypes was inferred. Among the totally nested clades, past fragmentation and/or long distance colonization was inferred. From these results, the nesting population of southwestern Cuba, although sharing a historical connection with the regional populations, constitutes a separate demographic unit that should be managed independently.

Key words: mtDNA control region; population genetic; phylogeography; *Chelonia mydas*; ASW, Cuba.

## ESTRUCTURA POBLACIONAL DE LA TORTUGA VERDE (*Chelonia mydas*) DEL GRAN CARIBE, BASADA EN SECUENCIAS DE DNA MITOCÓNDRICO, CON ÉNFASIS EN COLONIAS DEL SUROESTE DE CUBA.

## RESUMEN

La conservación efectiva de las Tortugas marinas depende del conocimiento de las interconexiones demográficas entre las poblaciones. Nosotros caracterizamos la variación genética dentro y entre dos colonias de anidación de tortuga verde (*Chelonia mydas*) en el suroeste de Cuba [N=28] y evaluamos estos datos en un contexto regional para comprender la contribución de cada colonia de anidación a la estructura genética de la especie, a partir de la secuenciación de 490 pb de la región de control del DNA mitocondrial. En las colonias cubanas los haplotipos pertenecieron al mismo linaje (II) y el 71% de éstos resultaron endémicos, pero con baja representatividad. Entre las colonias de anidación cubanas no se encontró estructura genética significativa. Sin embargo, cuando se comparan los datos con las demás colonias del Gran Caribe se encontró estructura genética significativa (AMOVA), con mayor variación dentro que entre las colonias de anidación. La diferenciación genética ( $F_{ST}$ ) estuvo positivamente correlacionada con la distancia geográfica entre las colonias de anidación. En la población cubana, un patrón de expansión súbita fue soportado por una distribución desigual observada. Sin embargo, El resultado de la Prueba de Fu rechazó el modelo estándar de neutralidad, probablemente como una consecuencia de migraciones recientes. Usando el análisis de clados anidados, se infirió un flujo de genes restringido y un aislamiento por distancia para los clados que contuvieron la mayoría de los haplotipos cubanos. Una fragmentación pasada y/ o una colonización a largas distancia fue inferida entre el total de clados anidados. A pesar de que la población de anidación del suroeste cubano comparte una conexión histórica con las otras poblaciones de la región, constituye una unidad demográfica separada que debe ser manejada independientemente.

Palabras claves: región de control del mtDNA; genética poblacional; filogeografía; *Chelonia mydas*; ASW, Cuba.

Green turtles (*Chelonia mydas*) are long-lived marine animals with complex life histories. Once hatchlings emerge from nests laid on sandy beaches, they enter a pelagic phase where prevailing currents extensively disperse, and effectively mix individuals from multiple rookeries (Bjorndal & Bolten, 2008). They spend several years in pelagic environments before recruiting as juveniles in neritic habitats and can henceforth move between various developmental habitats (Webster *et al.*, 2002; Bjorndal & Bolten, 2008) before reaching sexual maturity. Once turtles reach breeding status in foraging habitats, they embark on focused and extensive migrations through which individual populations segregate at individual natal sites for breeding; a phenomenon known as “philopatry” (Carr, 1975). This complicated life history means that they will be exposed to multiple threats during a very long period and over wide geographic ranges, thereby significantly compromising their conservation (Seminoff, 2002). A further consequence of this complex life history is that individual populations of this species are extremely difficult to monitor; thus, their distribution and genetic structure are only partially understood.

Populations of *C. mydas* are widely distributed in tropical and subtropical oceans and are listed as *endangered* globally (IUCN, 2006) on the basis of a historical declining trend of their numbers, caused mainly by historical overexploitation of various life stages and, more recently, incidental mortality in fishery bycatch (Seminoff, 2002). However, the conservation status varies strongly between ocean basins. Though examples of population decline do exist, some populations in the Atlantic have shown dramatic recoveries and significant sustained population increases in comparison to other regions (Seminoff, 2002). At least seven major nesting populations occur in the Atlantic with more than 500 females per year: Ascension Island (Mortimer & Carr, 1987); Trinidad Island (Moreira, 1995); Tortuguero (Bjorndal *et al.*, 1999); Bioko Island (Thomas *et al.*, 1999); Bijagos Archipelago (Barbosa *et al.*, 1998); Suriname (Schulz, 1982); southern Florida (Meylan *et al.*, 1994). The nesting population of Turkey highlights in Mediterranean (Kasperek *et al.*, 2001). Two major nesting populations occur in the Atlantic with less than 500 females per year: Aves Island (Lagueux, 2001); and the Yucatan peninsula in Mexico (López, 2000).

Various secondary rookeries remain that have not been adequately studied to determine their

conservation status (Seminoff, 2002). *Chelonia mydas* rookeries of Cuba are centrally located within the species' Greater Caribbean migratory circuits. Early chronicles reflect a historical abundance of nesting in the Cuban Archipelago, but because there is little scientific reporting on nesting levels in this area (Seminoff, *op. cit.*), the true status of these rookeries is unknown. Western Cuba is recognized nationally as an important nesting area with Cayo Largo del Sur in the Canarreos archipelago having more than 2,000 nests per season (Nodarse, unpublished data). Other important nesting areas include Cayo Real in the Cayería de San Felipe, with approximately 40 nests recorded during 15 days at the end of the 1998 breeding season (Ruiz *et al.*, 2003); El Guanabaco beach in southern Isla de la Juventud, with 98 nesting females tagged from 1989 to 1996 (Nodarse *et al.*, 2000); and the Guanahacabibes Peninsula, with 638 nests and 172 nesting females tagged during 2002 (Azanza *et al.*, 2003). Results from flipper tagging studies strongly indicate that Cuban sites constitute a central hub for regional populations that are in migration, or that use Cuban habitats as foraging and/or developmental areas (Moncada *et al.*, 2006). However, limited monitoring of Cuban rookeries and the lack of full analysis of their genetic makeup have hindered insight into their contributions to local (i.e. Cuban) and regional foraging habitats.

The genetic structure of green turtles is defined by the geographic distribution of critical habitats and dispersion and migratory patterns, in combination with the philopatric nature of the species. Precisely because of philopatry and natal homing, *C. mydas* populations are subdivided into geographically and genetically distinct breeding units that can be identified with mitochondrial DNA (mtDNA) sequences (Allard *et al.*, 1994). Mitochondrial DNA (mtDNA) is the molecular marker of choice to detect matrilineal population structure at various geographic scales (Bowen *et al.* 1992; Lahanas *et al.* 1994; Norman *et al.* 1994; Encalada *et al.* 1996; FitzSimmons *et al.* 1997) and provides important information on dispersion patterns, geographical variation, and phylogeny (Avice, 1986; Moritz *et al.*, 1987). While green turtle rookeries tend to be strongly differentiated from each other over timescale where demography and migration acts i.e. ecological timescales (e.g. large mtDNA haplotype frequency shifts), because of female natal homing behavior, they remain phylogenetically closely related (high similarities in mtDNA nucleotide sequences) with occasional

“mistakes” in natal homing that allow the occasional mixing of matriline.

Using mtDNA, Encalada *et al.* (1996) identified two major green turtle lineages in the Atlantic basin: western Caribbean and Mediterranean Sea (“Lineage II”); and eastern Caribbean, South Atlantic and western Africa (“Lineage I”). Within regions, significant genetic differentiation between rookeries is found amongst the majority of rookeries (Lahanas *et al.*, 1994). There are a few relevant exceptions (Encalada *et al.*, 1996), such as Mexico and Florida; Suriname and Aves Island; and Ascension Island and Guinea Bissau, which could be the result of current genetic flow or the outcome of relatively recent isolation events. Nevertheless, the degree of inter-population genetic distinctiveness among regional populations allows an evaluation of the extent that individual green turtle populations overlap at foraging grounds (Luke *et al.*, 2004; Bass *et al.*, 2006; Naro-Maciel *et al.*, 2007) through mixed stock analyses based on mtDNA sequences (Bolker *et al.*, 2007; Bjorndal & Bolten, 2008).

Considering the contrasting conservation status of *C. mydas* populations in the Atlantic and the pressing need to understand how each breeding unit contributes to the various habitats of the species, we determine the genetic identity of western Cuban rookeries and their genetic relationships to the remaining populations using mitochondrial DNA control region sequences, thereby contributing to previous phylogeographic studies in the Greater Caribbean (Allard *et al.*, 1994; Lahanas *et al.*, 1994, 1998; Encalada *et al.*, 1996). We also evaluate rookery demographic history to propose scenarios that explain the observed patterns of genetic diversity at present, and on an evolutionary timescale, as well as the degree of genetic contribution by western Cuban rookeries to regional foraging sites. Our data and analyses will contribute to the development of scientifically-based management plans for this species’ conservation within and outside Cuba.

## MATERIALS AND METHODS.

### Study area

The samples analyzed in this work were collected in Boba beach [21° 58'N; 83° 36'O] belonging to Cayo Real (Cayería San Felipe), and two others beaches belonging to Guanahacabibes Peninsula: Antonio beach [21° 91'N; 84° 65'O] and Caleta de los Piojos Beach [21° 82'N; 84° 87'O] (Fig. 1).

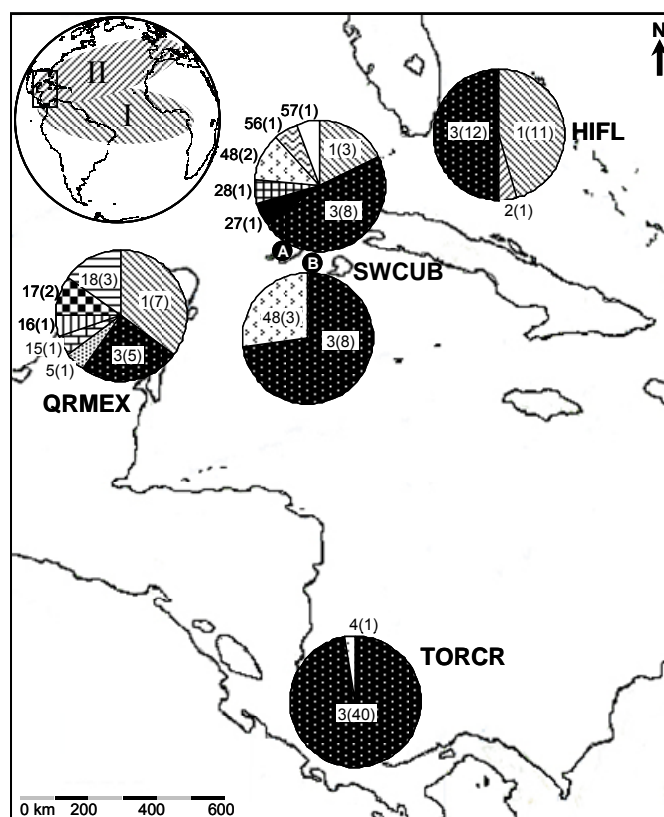


Fig. 1. Map showing sampling locations (by means of letter) and corresponding mtDNA haplotypes frequencies (absolute and relative frequencies by means of number in parenthesis and shades in the pies, respectively) of *C. mydas* rookeries in Greater Caribbean. The analysis was based on mtDNA control region (ca. 493 bp) haplotypes. Atlantic lineages: I – South Atlantic; II – Greater Caribbean and Mediterranean Sea. Nesting beaches: A – Guanahacabibes peninsula; B – Cayería San Felipe. Haplotypes represented by only number: A1 – 4 (Allard *et al.* 1994); A5 (Lahanas *et al.* 1994); A15 – 18 (Encalada *et al.* 1996); A27 and 28 (Bjorndal & Bolten, submitted to GenBank 2001); A48, 56 and 57 (Espinosa *et al.* submitted to GenBank 2003).

### Field and laboratory

Tissues samples were extracted from dead turtle embryos from different nests laid at two breeding sites in southwestern Cuba where a mark-recapture program existed (see Fig. 1). Eleven embryos were taken from Boba beach during August 1998 and 17 from the Guanahacabibes Peninsula during 2000. The Guanahacabibes samples consisted of three embryos from Antonio beach, and 14 from Caleta de los Piojos.

Since individual females have been observed nesting at more than one beach in this area both

during and between breeding seasons (Azanza, unpublished data), we combined samples from the two beaches (separated by *ca.* 25 km) for all analyses and they are referred to collectively as the “Guanahacabibes Peninsula” sample. Additionally both beaches belong to oneself geographical accident with similar characteristics of the landscape as of the type submerged plain of the insular platform subjected to a strong intern surf of the subtypes (Mateo, 1989): 1) with weak vegetable covering, on rock and carbonated sand and 2) *idem*, but only on rock. All samples were preserved in 90% ethanol at ambient temperature during the fieldwork and afterwards under refrigeration (4°C) until further processing.

DNA extraction was performed using the method of Hillis *et al.* (1996) and approximately 490 base pairs (bp) from the mtDNA control region were amplified using L15926 (Kocher *et al.*, 1989) and TCR6 (Norman *et al.*, 1994) primers. This primer combination generated amplicons with approximately 80 more bases at the 5' end (including portions of both the Thr and Pro tRNA sites) than the more commonly used LTCM1/HDCM1 primer pair of Allard *et al.* (1994). The PCR was performed in 50 µL reactions, containing 2 units of *Taq* polymerase, 1x PCR buffer II (Promega), 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 0.2 µM of each primer, together with 10-500 ng of genomic DNA. A program with an initial denaturalization step of 4 min at 94° C; followed by 40 cycles of 94°C for 1 min, 45°C for 45 s, 72°C for 1 min; and a 10 min final extension at 72°C was used.

PCR products were purified using High Pure PCR product purification kits (Roche) then cycle-sequenced (Sanger *et al.*, 1977) using Thermo Sequenase kits (Amersham) in both directions using P<sup>33</sup> delta-labeled L-primer. Sequencing products were separated through 6% polyacrylamide vertical electrophoresis, exposed to photosensitive plates for 48 hours and scored manually after development.

## Data Analysis

### Population structure

Sequences from southwestern Cuban rookeries were identified and characterized through alignments using Clustal X 1.81 (Thompson *et al.*, 1997) against green turtle reference haplotypes belonging to Lineage II (CM-A1, 2, 4, 13 – 18, Encalada *et al.* (1996) [GenBank accession numbers Z50124, Z50125, Z50126, Z50135 – 40];

A3, Allard *et al.* (1994) [M98394]; CM-A22, 26 – 31 and 34, Bjørndal & Bolten (submitted to GenBank 2001) [AF366251, AF366255 – 60, AF366263]. For this selection we considered the genetic distinctions between *C. mydas* lineages of the Atlantic and Indo-Pacific basins (Bowen *et al.*, 1992), and among the lineages II and I (Encalada *et al.*, 1996). Position for polymorphic sites (PSs) was assigned using the start of the mtDNA non-coding region as a reference point (Kumazawa & Nishida, 1999). Structural relationships between haplotypes were determined using a minimum spanning network tree (Rohlf, 1973). For further analyses, the 6 bp insert found in sequence CM-A48 was coded as a single change.

To ascertain genetic relationships among regional rookeries, we limited our analysis to those rookeries sharing at least one haplotype with the Cuban rookeries; *i.e.*, Tortuguero (Costa Rica) [N=41]; Quintana Roo and Cozumel Island (Mexico) [N=20]; and Hutchinson Island Florida (USA) [N=24] (Allard *et al.*, 1994; Encalada *et al.*, 1996; Lahanas *et al.*, 1998). We estimated genetic differentiation between population pairs using conventional  $F_{ST}$  through 10,000 permutations in Arlequin ver. 3.11 (Excoffier *et al.*, 2005). Assuming an island model, we used the  $F_{ST}$  values to estimate the absolute number of migrants per generation exchanged between pairs of populations ( $N_m$ ; Slatkin, 1991). Population haplotype frequencies were also compared using a  $\chi^2$  test of independence (Sokal & Rohlf, 1981), corrected with a sequential Bonferroni procedure (Rice, 1989) with 1,000 simulations (Roff & Bentzen, 1989), using the program CHIRXC (Zaykin & Pudovkin, 1993). In all the cases we recalculated statistics for rookeries in Encalada *et al.* (1996). In the case of the Tortuguero rookery, we chose to compare our results with those of Allard *et al.* (1994) and Lahanas *et al.* (1998) since their sample sizes were similar to ours and because the characteristic haplotype composition was not significantly affected when the sample size was incremented (Bjørndal *et al.*, 2005). The sample size was increased ten times. However, the contribution of CM-A3 continues being preponderant (91%) in the genetic composition in Tortuguero rookery. Nevertheless, other haplotypes of recent dispersion that appear in very low frequency increase notably the degrees of freedom in the statistical test. This trend could also be observed in other rookeries. Therefore, we prefer to use similar sample sizes for each rookery for the statistical analyses, and in particular, to estimate the phylogeographic relationships. Arlequin was also used with 10,000

random permutations to evaluate the hierarchical genetic structure using conventional  $F_{ST}$  values in AMOVA (Excoffier *et al.*, 1992) and with Mantel tests (Smouse *et al.*, 1986).

### Gene diversity

Gene (Nei, 1987) and nucleotide (Tajima, 1983; Nei, 1987) diversities were characterized after confirming that variation conformed to the standard neutral model as tested by Tajima's D statistic (Tajima, 1989 and 1996) and Fu's test (Fu, 1997) with 10,000 simulations (Stewart, 1977), implemented in Arlequin. Population effective sizes (Watterson, 1975) were estimated considering an average remigration interval of 3 years (Miller, 1997; Van Buskirk & Crowder, 1994) and a mutation rate of 1.2% *per* nucleotide site *per* million years (MY) *per* pair of lineages (Avise *et al.* 1998), using the methodology of Avise *et al.* (1992) and according to the data of Bowen *et al.* (1992), adapted to mtDNA sequences from Encalada *et al.* (1996). This mutation rate corresponded to the upper estimate of 3.0 MY and not to the average time proposed for the rise of the Panama isthmus (3.0-2.5 MY) according to Lundelius (1987) and used by Avise *et al.* (1992). We corrected the mutation rate with the average generation period (47 years; calculated according to Pianka, 1974) and used a sexual maturation average of 37.5 years (Limpus & Chaloupka, 1997; Bjørndal *et al.*, 2000) and an average reproductive life span of 19 Y (Chaloupka *et al.*, 2004). We calculated Theta according to the number of segregating sites and the nucleotide diversity using Arlequin.

Historical demographies were calculated from the mismatch distribution through the sudden expansion model (Rogers & Harpending, 1992) using Arlequin. The model provides rookery effective sizes before expansion and the time interval (in years) for the expansion associated with a confidence interval (CI) (Li, 1977) with alpha equal to 0.05. In the case where theta=0 at the beginning of the expansion, the effective size was calculated from the average CI for the statistic with the same alpha value. In these analyses the significance of Fu's Test was taken into consideration. The comparison of the effective and census population sizes in endangered species is a very important task, mainly if the populations of the species occupy separate patches of a subdivided habitat. The effective population size is an estimate of the size that should have a population according to its genetic diversity. In metapopulation, increasing  $F_{ST}$  will increase the

long-term effective population size (Nunney, 2000). However, when this estimate and the census size differ in a metapopulation, being frequently higher the second when differential productivity of the demes brings about differential contributions to the migrant pool (Nunney, 1999), the effective size of a metapopulation is reduced by increasing  $F_{ST}$  known as "interdemographic genetic drift".

### Phylogenetic relationships

Genetic relationships between haplotypes were illustrated by Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering (Sneath & Sokal, 1973) and Neighbor-Joining (NJ) (Saitou & Nei, 1987), using MEGA ver. 3.0 (Kumar *et al.*, 2004). Trees were rooted using haplotype CMU40436 from the Pacific *C. mydas* lineage (Dutton *et al.*, 1996) as outgroup. Only haplotypes corresponding to populations that shared at least one haplotype with Cuban rookeries were included in order to build a diagram of phylogenetic relationships among mtDNA haplotypes and nesting populations. Statistical supports for nodes were estimated by non-parametric bootstrapping (Felsenstein, 1985) with 10,000 pseudo-replicates. Divergence times among the haplotypes were also based on the upper estimated age for the Panamanian isthmus (Lundelius, 1987). Sequence divergences were estimated as distances within and among populations (Nei, 1987: formulas 10.19 and 10.20 respectively) and net number of nucleotide substitutions between two populations (Nei & Li, 1979). The net number of nucleotide substitutions was used to estimate divergence times among populations (Nei, 1987: formula 10.22).

### Phylogeographic relationships

Interpretation of distance trees was based on a Nested Clade Phylogeographic Analyses (NCPA) (Templeton *et al.*, 1987) of North Atlantic haplotypes but we also included the Lara (Cyprus) population because its haplotypes belong to the same lineage represented in Cuban rookeries. First, we calculated the maximum number of connections between pair-wise sequences according to a "parsimony" criterion (Templeton *et al.*, 1992), as well as the haplotype outgroup (Castelloe & Templeton, 1994). This process resulted in an unrooted dendrogram, with nine steps corresponding to the 95% limit of connections, using the program TCS ver. 1.21 (Clement *et al.*, 2000). Subsequently, we defined two series of hierarchically nested clades according



to Templeton *et al.* (1992) and Templeton & Sing (1993), which were statistically analyzed in terms of the relationships between the genealogy of haplotypes and straight-line geographic distance among the rookeries with program GEODIS ver 2.5 (Posada *et al.*, 2000). The clades with significant distance values were interpreted through the NCPA inference keys (Templeton, 2004).

## RESULTS.

### Population structure

All southwestern Cuba haplotypes belonged to Lineage II (Fig. 1). Three new haplotypes are endemic to Cuban rookeries [GenBank accession numbers AJ543730, AJ543732, and AJ543735], and have been designated CM-A48, 56 and 57 respectively, following the Archie Carr Center for Sea Turtle Research nomenclature. Two other haplotypes (CM-A27 and 28) [AF366256 and 57; Bjorndal and Bolten, submitted to GenBank 2001] found in the Guanahacabibes Peninsula rookery also be endemic. Only one of the new endemic haplotypes (CM-A48), reported for the foraging areas of Florida (Bagley, 2003), was shared with both southwestern Cuba rookeries.

Phylogenetic analyses for the five haplotypes indicate they could be interconnected in a network with one or two changes between neighboring haplotypes, and associated to the two most widely distributed and best represented haplotypes (CM-A1 and 3; *i.e.*, diversification centers) within the Lineage II (Fig. 2). Analysis to determine the most parsimonious routes demonstrated that the number of changes that exist between haplotypes around the CM-A1 and 3 haplotypes was independent of the diversification center ( $\chi^2_{(4)} = 5.67$ ,  $p = 0.24$ ). When we made a similar analysis to determine the changes dependent upon the position (internal or external) of the haplotypes, no correlation was found for the number of changes between internal haplotypes and the diversification centers ( $\chi^2_{(4)} \approx 0$ ). However, we found dependence for the numbers of changes that exist between the external haplotypes and the diversification centers ( $\chi^2_{(4)} = 9.52$ ,  $p = 0.03$ ). It was more frequent one change regarding CM-A3 and two regarding CM-A1. A third diversification center appears to exist around haplotype CM-A13 of the Lineage II.

Approximately 94% of the total substitutions (31) were transitions with similar numbers involving purines and pyrimidines. The remaining substitutions were transversion at PS 365 and a

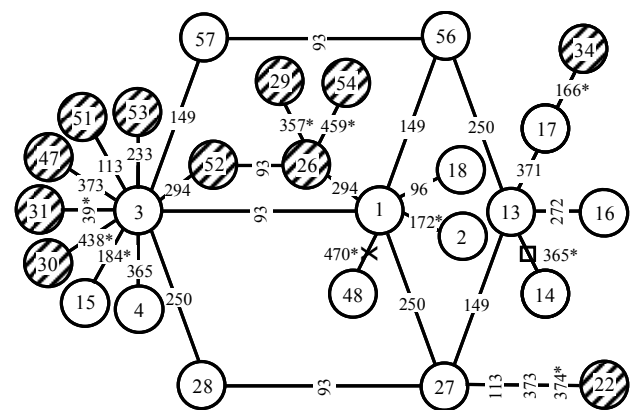


Fig. 2. Minimum-spanning tree based on pairwise nucleotide differences between mtDNA haplotypes of *C. mydas* from Lineage II. The analysis was based on mtDNA control region (ca. 493 bp) haplotypes. Open circles = the haplotype *i*, shaded circles = haplotypes of unknown origin; numbers outside of the circles = mutations, square: transversions, x = an addition/deletion (CAATGG), \* = a diagnostic change. Haplotypes: CM-A1 – 4 (Allard *et al.*, 1994); CM-A13 – 18 (Encalada *et al.*, 1996); CM-A22, 26 – 31 y 34 (Bjorndal & Bolten, submitted to GenBank 2001); CM-A48, 56 y 57 (Espinosa *et al.* submitted to GenBank 2003); CM-A51 – 53 (Formia, submitted to the Archie Carr Center); 5CM-A54 (Meylan, submitted to the ARchie Carr Center).

six bp (CAATGG) insertion at PS 470. Thirty-two percent of these substitutions represented diagnostic changes for haplotypes. The aforementioned transversion and the insertion were for CM-A14 and 48, respectively, whereas the transitions (8) at PS 39, 166, 172, 184, 357, 374, 438, and 459 were found in haplotypes CM-A31, 34, 2, 15, 29, 22, 30, and 54, respectively.

Among the Greater Caribbean haplotypes, we found a maximum of 17 PSs. These PSs were composed of 15 transitions, a transversion at position 284, and the aforementioned insertion (Table 1). The transversion belongs to haplotype CM-A5, which is rare in the Quintana Roo rookery (Encalada *et al.*, 1996). This change and the transitions at positions 431 and 433 constituted diagnostic PSs for Atlantic lineages (between I and II). In samples from southwestern Cuba, only four polymorphic sites were detected (PSs 93, 149, and 250; and the insertion).

No significant genetic differentiation was found between the two southwestern Cuban rookeries (Table 2). We did not find significant genetic structure between the Guanahacabibes Peninsula

Table 1. Polymorphism in 5' half of the mtDNA control region (ca. 493 bp) of *C. mydas* haplotypes from American Mediterranean nesting areas. We designated the corresponding number to each polymorphic site using the first base of the mtDNA control region (Kumazawa & Nishida, 1999).  $\pm$  = addition/ deletion of CAATGG. Haplotypes: CM-A1 - 4 (Allard *et al.*, 1994); CM-A5 (Lahanas *et al.*, 1994); CM-A15 - 18 (Encalada *et al.*, 1996); CM-A27 and 28 (Bjorndal & Bolten, submitted to GenBank 2001); CM-A48, 56 and 57 (Espinosa *et al.*, submitted to GenBank 2003).

| haplotype | Polymorphic sites |    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----------|-------------------|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|           | 93                | 96 | 133 | 149 | 172 | 184 | 250 | 272 | 284 | 365 | 367 | 371 | 410 | 431 | 433 | 470 |
| CM-A1     | C                 | A  | G   | G   | A   | T   | T   | C   | T   | C   | G   | G   | T   | A   | G   | -   |
| CM-A2     | C                 | A  | G   | G   | G   | T   | T   | C   | T   | C   | G   | G   | T   | A   | G   | -   |
| CM-A3     | T                 | A  | G   | G   | A   | T   | T   | C   | T   | C   | G   | G   | T   | A   | G   | -   |
| CM-A4     | T                 | A  | G   | G   | A   | T   | T   | C   | T   | T   | G   | G   | T   | A   | G   | -   |
| CM-A5     | C                 | G  | A   | G   | A   | T   | C   | C   | G   | C   | A   | G   | C   | G   | A   | -   |
| CM-A15    | T                 | A  | G   | G   | A   | C   | T   | C   | T   | C   | G   | G   | T   | A   | G   | -   |
| CM-A16    | C                 | A  | G   | A   | A   | T   | C   | T   | T   | C   | G   | G   | T   | A   | G   | -   |
| CM-A17    | C                 | A  | G   | A   | A   | T   | C   | C   | T   | C   | G   | A   | T   | A   | G   | -   |
| CM-A18    | C                 | G  | G   | G   | A   | T   | T   | C   | T   | C   | G   | G   | T   | A   | G   | -   |
| CM-A27    | C                 | A  | G   | G   | A   | T   | C   | C   | T   | C   | G   | G   | T   | A   | G   | -   |
| CM-A28    | T                 | A  | G   | G   | A   | T   | C   | C   | T   | C   | G   | G   | T   | A   | G   | -   |
| CM-A48    | C                 | A  | G   | G   | A   | T   | T   | C   | T   | C   | G   | G   | T   | A   | G   | +   |
| CM-A56    | C                 | A  | G   | A   | A   | T   | T   | C   | T   | C   | G   | G   | T   | A   | G   | -   |
| CM-A57    | T                 | A  | G   | A   | A   | T   | T   | C   | T   | C   | G   | G   | T   | A   | G   | -   |

Table 2. Pairwise comparison of *C. mydas* rookeries from American Mediterranean, using mtDNA control region (ca. 493 bp) haplotypes. Above diagonal: pairwise haplotype frequency comparisons based on  $\chi^2$  statistics corrected with the Bonferroni procedure using the Monte Carlo randomization method, degrees of freedom (in parentheses), and probability (/ value). Below diagonal: absolute numbers for exchanged migrants and  $F_{ST}$  probability (/ value), based on non-parametric exchanges. The data of the rookeries with superscript 1, 2, and 3 belonged to Encalada *et al.* (1996), Allard *et al.* (1994), and Lahanas *et al.* (1998), respectively.

| Locality                                  | Guanahacabibes<br>(Cuba) | San Felipe<br>(Cuba)          | southwestern<br>Cuba | Florida <sup>1</sup><br>(USA)   | Quintana Roo <sup>2</sup><br>(Mexico) | Tortuguero <sup>2,3</sup><br>(Costa Rica) |
|---|--------------------------|-------------------------------|----------------------|---------------------------------|---------------------------------------|---|
| Guanahacabibes<br>(Cuba)                  |                          | 6.20 <sub>(6)</sub> /<br>0.43 |                      | 11.51 <sub>(7)</sub> /<br>0.04  | 16.16 <sub>(11)</sub> /<br>0.04       | 25.82 <sub>(7)</sub> /<br><0.001          |
| San Felipe<br>(Cuba)                      | 16.23<br>0.03 / 0.22     |                               |                      | 12.73 <sub>(3)</sub> /<br>0.004 | 17.55 <sub>(7)</sub> /<br>0.002       | 12.03 <sub>(2)</sub> /<br>0.007           |
| southwestern<br>Cuba                      |                          |                               |                      | 14.92 <sub>(7)</sub> /<br>0.004 | 23.69 <sub>(11)</sub> /<br>0.002      | 21.60 <sub>(7)</sub> /<br><0.001          |
| Florida <sup>1</sup><br>(USA)             | 14.36<br>0.03 / 0.18     | 1.95<br>0.20 / 0.01           | 5.06<br>0.09 / 0.03  |                                 | 12.51 <sub>(7)</sub> /<br>0.03        | 25.37 <sub>(3)</sub> /<br><0.001          |
| Quintana Roo <sup>2</sup><br>(Mexico)     | 13.16<br>0.04 / 0.12     | 1.86<br>0.21 / 0.001          | 4.03<br>0.11 / 0.006 | 12.54<br>0.04 / 0.14            |                                       | 19.96 <sub>(7)</sub> /<br><0.001          |
| Tortuguero <sup>2,3</sup><br>(Costa Rica) | 0.86<br>0.37 / <0.001    | 1.03<br>0.33 / 0.005          | 3.48<br>0.12 / 0.02  | 0.56<br>0.47 / <0.001           | 0.44<br>0.53 / <0.001                 |   |

rookery and the rookeries of the continent (Florida and Quintana Roo). However, the probability associated to the  $\chi^2$  probability was significant although the higher confidence interval was similar to the critical value of non-significance. Our analysis suggested a flow of migrants on the order of dozens of individuals per generation. However, the San Felipe rookery had significant genetic structure with the rookeries of the continent. The Tortuguero rookery was clearly significantly differentiated with respect to the other Greater Caribbean rookeries.

The genetic structure found was confirmed by a significant  $F_{ST}$  in all hypotheses (Table 3), with a variation percentage almost four times higher among than between rookeries. The 30% of the PS integrated by the non-diagnostic *loci* (93:  $F_{ST}=0.36$ ,  $p<0.001$ ; 96:  $F_{ST}=0.18$ ,  $p=0.004$ ; 149:  $F_{ST}=0.08$ ,  $p=0.02$ ; and 250:  $F_{ST}=0.12$ ,  $p=0.007$  among haplotypes, and the diagnostic *locus* 470 ( $F_{ST}=0.18$ ,  $p<0.001$ ) of the endemic haplotype CM-A48 from southwestern Cuban rookeries contributed significantly to this variation. When we grouped the samples of the southwestern Cuban rookeries, we obtained the same level of significance and a similar percentage of variation within the rookeries. In this hypothesis (number 2), the  $F_{SC}$  and the  $F_{CT}$  were non significant. However, when we grouped the rookeries of southwestern Cuba with the Florida Peninsula rookeries (Hypothesis 3), the  $F_{SC}$  became significant, as a consequence of the significant contribution of the diagnostic PS 470 ( $F_{SC}=0.29$ ,  $p=0.01$ ). Consequently, we accepted hypothesis 2.

The degree of genetic differentiation ( $F_{ST}$ ) for pairs of rookeries was correlated with the net number of nucleotide substitutions ( $r=0.78$ ,  $p=0.01$ ) and with the geographical distance ( $r=0.83$ ,  $p=0.003$ ) (Fig. 3). The net number of nucleotide substitutions and the geographical distances were not significantly correlated ( $r=0.41$ ,  $p=0.24$ ).

### Gene diversity

The nesting population of southwestern Cuba was characterized by a high percentage of haplotype endemism (71.43%), although we found a moderate percentage of individuals with endemic haplotype (32.14%) in relation to the other nesting populations of the species. Within southwestern Cuba, the Guanahacabibes Peninsula rookery presented a genetic diversity approximately double that of the San Felipe rookery (Table 4). We identified only two haplotypes in the San Felipe

rookery that had a genetic diversity value similar to that of the Florida nesting population. Although the haplotype diversity of the Guanahacabibes rookery was as high as that reported for Quintana Roo (Encalada *et al.* 1996) with a similar number of haplotypes, the nucleotide diversity was approximately half and the number of segregating sites was almost one-quarter that of the Mexican nesting population. This proportion remained even when we combined the data from the two southwestern Cuba rookeries.

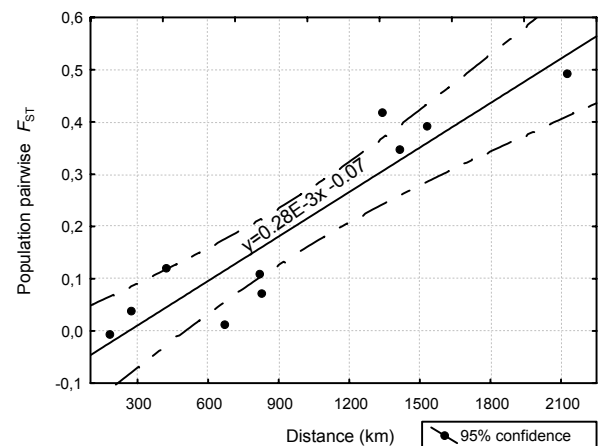


Fig. 3. Linear regression between the genetic differentiation ( $F_{ST}$  estimated from mtDNA control region (ca. 493 bp) haplotypes) versus the geographic distance for pairwise of *C. mydas* rookeries from Greater Caribbean. Continuous line: curves of linear regression; discontinuous line: confidence interval.

The effective sizes of the nesting populations estimated from theta, either from the number of segregating sites or from the nucleotide diversity, were of the same order of magnitude (Table 4). The exception was the Tortuguero nesting population, where according to theta, the effective size was approximately one order of magnitude higher than the number of segregating sites. The estimated effective size considering the nucleotide diversity of the Guanahacabibes Peninsula rookery according to theta was three times higher than that of the current female census abundances. The Quintana Roo nesting population's estimated effective size considering the nucleotide diversity was five times higher. In the other two nesting populations, the disproportion was in the opposite direction: the female census size of the Florida nesting population had twice the effective size, whereas the Tortuguero nesting population had two thousand times the effective population size. Only the Florida nesting population had an estimated effective size equivalent to the historical female census size.



Table 3. Hierarchical analyses of molecular variance under three hypothesized levels of geographic structure of *C. mydas* nesting populations from American Mediterranean region. The analyses were based on mtDNA control region (ca. 493 bp) haplotypes.  
P: probability value.

| Variance component  | Observed partition<br>Variance | % total | <i>F</i> statistics | p      |
|---|--------------------------------|---------|---------------------|--------|
| <b>I. Hypothesized structure: panmictic population</b>  |                                |         |                     |        |
| Between populations   | 0.07                           | 25.55   | 0.26                | <0.001 |
| Within populations  | 0.22                           | 74.45   |                     |        |
| <b>II. Hypothesized structure: metapopulation, where the rookeries of southwestern Cuba are a panmictic population</b>                        |                                |         |                     |        |
| Between regions   | 0.06                           | 20.20   | 0.20                | 0.30   |
| Between populations/ regions  | 0.02                           | 5.93    | 0.07                | 0.22   |
| Within populations  | 0.22                           | 73.87   | 0.26                | <0.001 |
| <b>III. Hypothesized structure: metapopulation, where the rookeries of southwestern Cuba and Florida Peninsula are a panmictic population</b> |                                |         |                     |        |
| Between regions   | 0.05                           | 16.74   | 0.17                | 0.20   |
| Between populations / regions   | 0.03                           | 11.02   | 0.13                | 0.04   |
| Within populations  | 0.22                           | 72.24   | 0.28                | <0.001 |

Table 4. Measures of genetic diversity and demographic indexes of rookeries/ nesting populations of *C. mydas* from American Mediterranean. The analyses were based on mtDNA control region (ca. 493 bp) haplotypes. The data of the rookeries with superscript 1, 2, and 3 belonged to Encalada *et al.* (1996), Allard *et al.* (1994), and Lahanas *et al.* (1998), respectively. The effective size of female was calculated by reproductive season. The census values of female by reproductive season were obtained starting from Seminoff (2002), except for Guanahacabibes rookery (Ibarra, 2005).  
a, parameter of the sudden expansion model without the contribution of the haplotype A5; CI, confidence interval for an alpha equal to 0.05; *D*, Tajima's test; *F<sub>s</sub>*, Fu's test; *H*, haplotypes number;  $\hat{H}$ , haplotype diversity; *N*, sample size; *N<sub>e(t)</sub>*, estimated effective size of the rookery; *N<sub>e(f)</sub>*, female's census size of rookery; *N* ( $\hat{\theta}_0$ ), census size before the expansion; *p*, probability; *ps*, polymorphic sites; *S*, squares sum; *Sd*, standard deviation; *t*, time where the expansion expressed in 1,000 years began (KY);  $\hat{\theta}$ , theta estimator: subscript *S* y *0* correspond to the number of segregating sites and the initial sizes of the population during the expansion, respectively;  $\hat{\pi}$ , nucleotide diversity;  $\tau$ , time of the expansion measured in  $1/2u$  generations; !, the procedure of the square minim to adjust the mismatch distribution and the observed distribution did not converge after the 1,800 steps.

| Collection region  | <i>N</i> | <i>H</i> | $\hat{H} \pm Sd(\hat{H})$ | $\hat{\pi}_n \pm Sd(\hat{\pi}_n)$ | <i>ps</i> | <i>D</i> ( <i>p</i> )/<br><i>F<sub>s</sub></i> ( <i>p</i> ) | <i>N<sub>e(t)</sub></i><br>$\hat{\theta}_S / \hat{\theta}_\pi$ | <i>N<sub>e(f)</sub></i><br>Historical/<br>current? | $\tau$                | Mismatch distribution<br>(sudden expansion) |                              |                 |
|--|----------|----------|---------------------------|-----------------------------------|-----------|---|--|--|-----------------------|---|------------------------------|-----------------|
| Guanahacabibes<br>(Cuba in 2000)                           | 17       | 7        | 0.7647 ± 0.0943           | 0.0024 ± 0.0018                   | 4         | -0.02 (0.48)/<br>-3.34 (0.002)                              | 709/ 708   | / 213  | !                     |   |                              |                 |
| San Felipe<br>(Cuba in 1998)                               | 11       | 2        | 0.4364 ± 0.1333           | 0.0018 ± 0.0015                   | 2         | 0.85 (0.81)/<br>2.01 (0.83)                                 | 409/ 525   | ?  | 2.9                   | 0.16<br>(0.02)                              | -                            | -               |
| Southwestern Cuba  | 28       | 7        | 0.6481 ± 0.0890           | 0.0022 ± 0.0017                   | 4         | 0.07 (0.58)/<br>-2.67 (0.03)                                | 616/ 635   | ?  | 1.5                   | 0.03<br>(0.13)                              | 131<br>(21-312)              | 62              |
| Florida Peninsula <sup>2</sup><br>(USA in 1986 & 90)       | 24       | 3        | 0.5616 ± 0.0468           | 0.0012 ± 0.0011                   | 2         | 0.28 (0.72)/<br>0.29 (0.51)                                 | 408/ 364   | 366/ 759   | 0.8                   | 0.04<br>(0.002)                             | -                            | -               |
| Quintana Roo <sup>1</sup><br>(Mexico in 1993)              | 20       | 7        | 0.8158 ± 0.0575           | 0.0051 ± 0.0032                   | 14        | -1.36 (0.08)/<br>-0.56 (0.38)                               | 2,364/<br>1,500  | 82/ 298  | !<br>1.8 <sup>a</sup> | 0.02 <sup>a</sup><br>(0.22)                 | 154 <sup>a</sup><br>(40-335) | 49 <sup>a</sup> |
| Tortuguero <sup>2, 3</sup><br>(Costa Rica in<br>1990 & 96) | 41       | 2        | 0.0488 ± 0.0459           | 0.0001 ± 0.0002                   | 1         | -1.12 (0.14)/<br>-1.47 (0.03)                               | 140/ 29  | 25,000/<br>58,000                                  | 3.0                   | 0<br>(0.16)                                 | 253<br>(37-254)              | 4               |

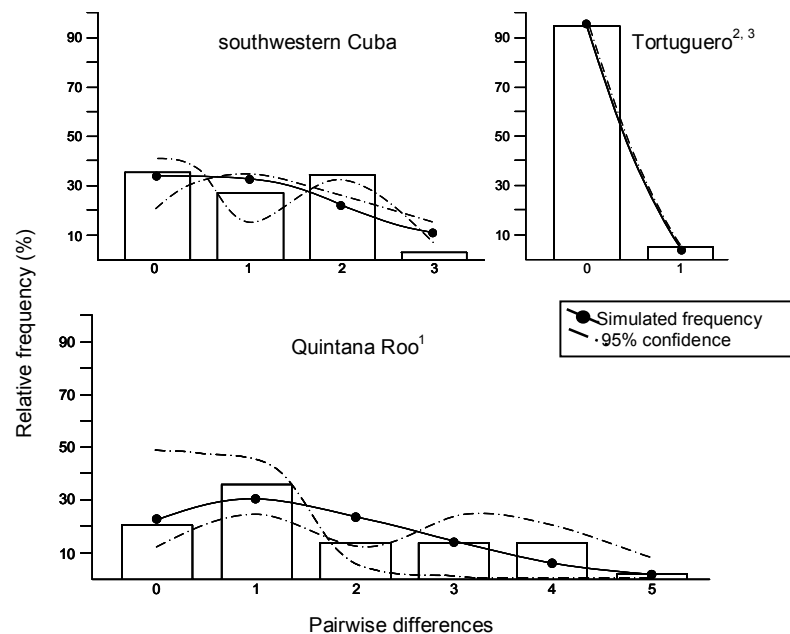


Fig. 4. Mismatch distributions based on mtDNA control region sequences (ca. 493 bp) of *C. mydas* nesting populations from Greater Caribbean. N with superscript 1, 2 and 3 are as Encalada et al. (1996), Allard et al. (1994) and Lahanas et al. (1998) respectively. The mismatch distribution represented in the Mexican (Quintana Roo) nesting population excludes the haplotype CM-A5.

The mismatch distribution test was run on the southwestern Cuba and Tortuguero nesting populations (Fig. 4). In both cases the curve was unimodal, with a mode of zero differences. In the southwestern Cuba nesting population, and contrastingly, in the Tortuguero nesting population, the expansion would appear to have occurred twice as recently and started with a small effective size, although the confidence intervals of the expansion time were overlapped to some degree. (see Table 4). Nevertheless, this result did not agree with those from Fu's test. (see Table 4). This test is sensitive to demographic expansions, considering that the significance at the 5% level is produced for probability  $<0.02$  (Excoffier et al., 2005). When the least squares procedure was analyzed to adjust for the observed and expected mismatches for the southwestern Cuba rookeries, we found no convergence in either the Guanahacabibes Peninsula rookery or in the Quintana Roo nesting population. When the contribution of haplotype CM-A5 was eliminated in the Quintana Roo nesting population, the pattern of sudden expansion was supported by the non-significance of the mismatch distribution test. However, Fu's test did not show statistical significance ( $F_s = -0.80$ ;  $p = 0.29$ ). For the San Felipe rookery, the pattern of sudden expansion was not

supported, because the deviation of the sum of squares was significant, as in the Florida nesting population.

### Phylogenetic relationships

The phylogenetic relationships among the haplotypes determined from the nesting populations, and estimated using distance methods, only identified two groups (Fig. 5). One included the haplotype CM-A5, belonging to the Lineage I (Encalada et al., 1996). The other included all the haplotypes of the Lineage II, whose topology changed in correspondence with the small bootstrap on each reconstruction. The majority of the haplotypes diverged from a common ancestor between 180,000 and 93,000 years ago, excepting the haplotype CM-A5, which appears to have diverged earlier, on the order of 800,000 years ago.

Among the populations, three groups were recognized whose divergences were at least three times more recent than that the divergences determined (see Fig. 5). The Tortuguero nesting population separated from the rest nesting populations with a  $dA = 0.06\%$ , from which we estimated a divergence time on the order of 30,000 years. The Quintana Roo nesting population

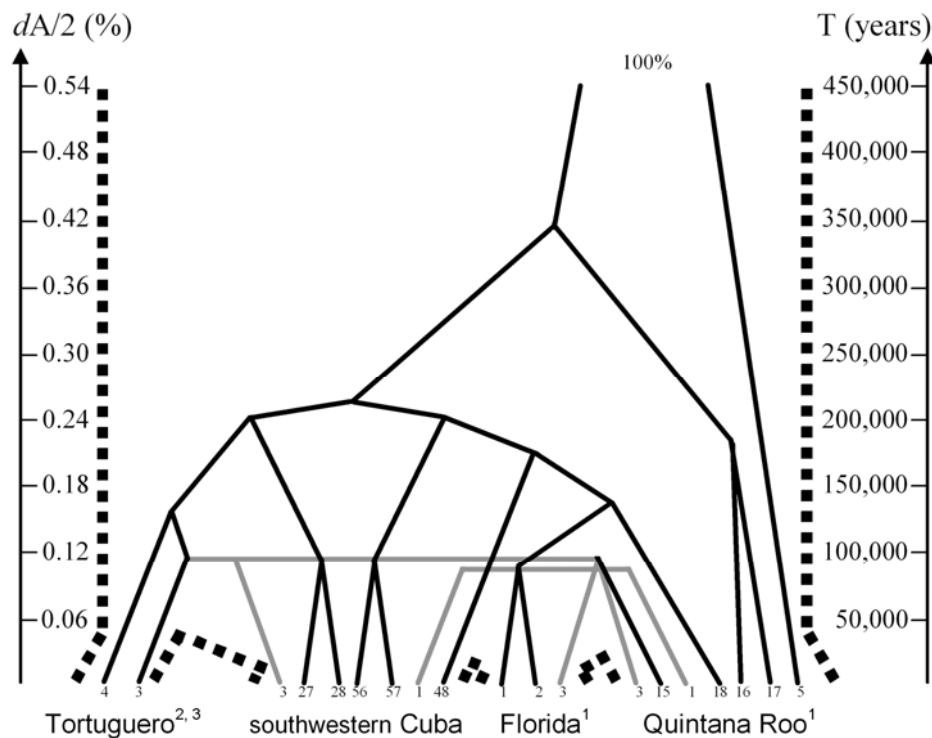


Fig. 5. Diagram of phylogenetic relationships among mtDNA haplotypes and nesting populations of *C. mydas* from Greater Caribbean, estimated from reconstruction methods based on distance matrix based on mtDNA control region sequences (ca. 493 bp). The reconstruction was determined by the net number of nucleotide substitutions between two populations (Nei & Li, 1979). Only bootstrap percentage greater than 50% are shown. Continuous, and discontinuous lines represent the relationships among haplotypes, and nesting populations respectively. Dark and gray lines, represent no sharing and sharing haplotypes among nesting population, respectively. The data of the rookeries with superscript 1, 2, and 3 belonged to Encalada *et al.* (1996), Allard *et al.* (1994), and Lahanas *et al.* (1998), respectively. Haplotypes: CM-A1 – 4 (Allard *et al.*, 1994); CM-A5 (Lahanas *et al.* 1994); CM-A15 – 18 (Encalada *et al.*, 1996); CM-A27 y 28 (Bjorndal & Bolten, submitted to GenBank 2001); CM-A48, 56 y 57 (Espinosa *et al.* submitted to GenBank 2003).

separated from the southwestern Cuba and Florida nesting populations by a *dA* two and a half times less than that of the divergence from the Tortuguero nesting population. Thus, we estimated the time of divergence of the Quintana Roo nesting population at approximately 11,000 years ago. Southwestern Cuba and Florida nesting populations appear to have separated by a *dA* twice as small as the *dA* which separated Quintana Roo from them (see Fig. 5). This distance represents only approximately 5,000 years.

### Phylogeographic relationships

In the NCPA, the nesting of clades (0 – step) resulted in haplotype groupings with different origins, except in clades 1 – 3 and 1 – 4, whose haplotypes are endemic to southwestern Cuba (Fig. 6A). In the most internal clade (1 – 1), the second best-distributed and best-represented haplotype

(CM-A1) of the lineage occurred with other endemic haplotypes of the southwestern Cuba, Florida, and Quintana Roo nesting populations. This haplotype was recognized as ancestral to the Lineage II. Clade 1 – 2 had similar representation. In this clade, the most widely distributed and best-represented haplotype (CM-A3) occurred with other endemic haplotypes of the Quintana Roo and Tortuguero nesting populations. The endemic haplotypes of the Quintana Roo and the Lara nesting populations were grouped in the remaining clade level (1 – 5).

Significant geographical associations were observed for the clades 1-1; 1-2; and 1-5 (Fig. 6B). In clade 1 – 1, we found large distances for the second-most widely distributed and represented haplotype inside the clade and within the nested clade, small distance for the haplotype CM-A48 inside the clade, and long average distance

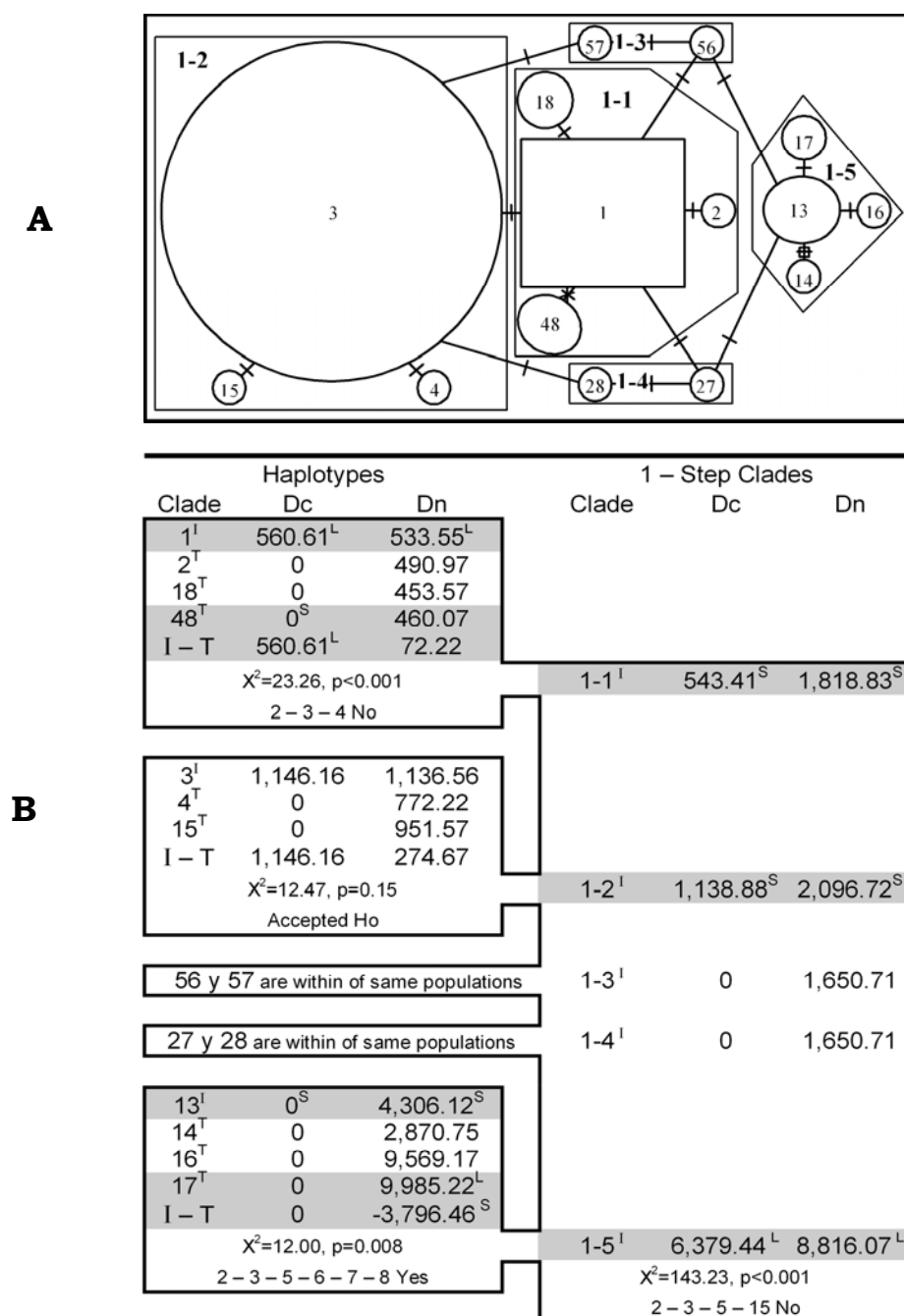


Fig. 6. Phylogeographic analysis of nested clades of the *C. mydas* Lineage II, based on on mtDNA control region sequences (ca. 493 bp). A) Minimum spanning tree of haplotypes with design of nested clades. B) Results from the NCPA. Square: ancestral haplotype; circle: i haplotype; -: a mutation, - with a square: a transversion and with an asterisk: an addition (CAATGG); fine lines: relationships among haplotypes mediated by a mutation; dark lines: 1 step among the clades, equivalent to the total of the cladogram. The size of the geometric figures is proportional to the magnitude of the haplotype. Dc and Dn: average distances of the clade and of the nested clade respectively; I and T: internal or external position of the clade; I – T: average distances between interior minus tip clades within a nesting clade; superscript S and L: indicate that the distance is significantly small or large. Under each rejected Ho are referred numbers corresponding to the use of the biological inference key according to Templeton (2004). Haplotypes: CM-A1 – 4 (Allard *et al.*, 1994); CM-A13 – 18 (Encalada *et al.*, 1996); CM-A27 y 28 (Bjorndal & Bolten, submitted to GenBank 2001); CM-A48, 56 y 57 (Espinosa *et al.* submitted to GenBank 2003).

between interior minus tip clades within a nesting clade. Accordingly, we inferred a restricted flow with isolation by distance from the significance of distances. Also in clade 1 – 5, we inferred a restricted genetic flow within the space with some dispersion which includes long distances around intermediate areas not occupied by the species or during past genetic flow followed by extinctions in intermediate populations. We made this inference based on the significance of the small distances of the haplotype CM-A13 inside the clade and from the nested clade, large distances for the haplotype CM-A17 in the nested clade, and small average distances between interior minus tip clades within a nesting clade. In clade 1 – 2, the null hypothesis of no geographical association was accepted. Among total nested clades (clades of 1 – step), we inferred fragmentation and/or colonization or movements at long distances, from the significance of small distances inside clades 1 – 1 and 1 – 2 and within these nested groups, and large distances inside clade 1 – 5 and in this nested group.

## DISCUSSION.

The present paper used only 28 new individuals to characterize genetic structure, phylogenetic and phylogeographic relationships in rookeries from southwestern Cuba. Despite this being a small sample size, it is equal to, or larger than the sample size used by other authors (Bowen *et al.*, 1992; see Table 1 p 868; Encalada *et al.*, 1996; Dethmers *et al.*, 2006) in more distant nesting areas.

### Population structure and gene diversity

Based on our analysis of genetic structure of *C. mydas* in southwestern Cuba rookeries, we conclude that they constitute a single panmictic breeding population. When we conducted a structure analysis between these colonies and the colonies of the remaining Greater Caribbean rookeries, we found that they were genetically distinct.

The incongruence between  $\chi^2$  test and  $F_{ST}$  that also was found by Encalada *et al.* (1996) for the comparison of genetic structure between Florida and Quintana Roo rookeries was solved with the application of an AMOVA. The first two methods estimate the differences between groups based on the absolute frequencies, and of these and the divergence between sequences, respectively. The third method also considers the variance

components, assuming as  $H_0$  that the samples represents a global population's sketch whose variation is due to a random sampling to the construction of the populations. The results of AMOVA test proved hypothesis number two, although  $F_{CT}$  was not significant. For this hypothesis the regions are real but the populations inside them are not, when individuals are exchanged inside regional groups without consider the population ( $F_{SC}$ ,  $p > 0.05$ ). For the hypothesis number three the regions are not real because the populations inside them are real, when individuals are exchanged inside regional groups without consider the population ( $F_{SC}$ ,  $p < 0.05$ ). That is why, this hypothesis is rejected. Regional differentiation is more apparent when the degree of difference between haplotypes is taken into account, in keeping with the observation that molecular distances are larger for pairs of haplotypes drawn from different regions than from the same region (Excoffier *et al.*, 1992).  $F_{CT}$  accuracy diminishes because the haplotypes of the rookeries in the Greater Caribbean belong to the same lineage, and because few changes exist among them. Additionally, these rookeries share the most widely distributed and best represented haplotype. For these reasons, we did not consider the  $F_{CT}$  index in hypothesis 2 and 3.  $\chi^2$  test gives expected answer contrary to  $F_{ST}$ , although the first only considers the number and frequency of haplotypes. In the case of the Guanahacabibes Peninsula rookery in relation to the nesting populations from Florida and Quintana Roo, the averaged coalescence time of two genes drawn from the same population is near the coalescence time for two comparable genes drawn in two populations. This result was a consequence of the most-represented haplotypes (CM-A1 and 3) being shared among rookeries, and the endemic remaining haplotypes appearing in low frequency in rookeries. Therefore, the  $F_{ST}$  is non-significant. Population genetic structure was also found among most of the South Atlantic (Encalada *et al.*, 1996; Formia *et al.*, 2006) and the IndoPacific (Norman *et al.*, 1994; Dethmers *et al.*, 2006) rookeries.

Considering the correlation that we obtained between pairwise  $F_{ST}$  and the geographical distance between rookeries, and the pattern of ecological distribution of the *C. mydas* in the nesting areas (a clumped distribution), the best-fitting gene flow model is the stepping stone model. This model explains the appearance of highly significant levels of structure among more distant rookeries, and clarifies the rookery and population concepts. The existence of discrete populations (demes) with

diverse ranks of structure is a consequence of isolation by barriers to dispersion; *e.g.*, distance associated with natal homing. This metapopulation is a system of demes, in patches with non-balanced demes in accordance with Harrison's classification (1991). Among demes patches, gene flow is a function of geographic distance, extirpation, and recolonization events in historical and ecological time. In the non-balanced demes, gene flow occurred during historical times as a result of colonization events. If recolonization occurred and affected the genetic structure of the Greater Caribbean's nesting population, the event(s) would have been before the lineage diversification. Our interpretation is consistent with results of the genetic diversity analysis (high endemism and low-to-moderate representation in the derivate haplotypes) and with the phylogeny of nested clades analysis (evidence of colonizations, extirpations, and recolonizations in a scenario of historical habitat fragmentation).

The high haplotype endemism in the southwestern Cuba nesting population is the consequence of natal homing behavior. This behavior is reinforced by insularity, by the emergence of the island during interglacial periods before the time interval in which the lineages of *C. mydas* diverged (last third of the Pleistocene, before the last glaciation), as well as by the maintenance of conditions necessary for nesting success during the glaciations. The moderately high haplotype diversity in San Felipe rookery (though it only shares two haplotypes with the Guanahacabibes Peninsula rookery) provides evidence that these keys constituted a sink, despite Guanahacabibes Peninsula and Cayería San Felipe rookeries being separated by ca. 150 km. Based on the results of FitzSimmons *et al.* (1997) and Encalada *et al.* (1996), Chassin-Noria *et al.* (2004) concluded that natal homing only operates on great geographical scales. In contrast, Peare & Parker (1996), using multilocus minisatellites, found significant negative correlation between the genetic similarity among pairs of nesting females, and the distance between their nests, both within and between seasons.

The demographic fluctuations only occurred in a few nesting populations during the Pleistocene; much earlier than the last glaciation. Consequently, the current distribution and representativeness of some *C. mydas* nesting populations could be much older than reported by Encalada *et al.* (1996). The non-correspondence in our results between Fu's test and the sudden

expansion model does not provide guidance on how to study the historical demography of the nesting populations. The distribution of the observed number of differences among haplotype pairs through the sudden expansion model (Rogers & Harpending, 1992) is less powerful than the Fu's test (Ramos-Onsins & Rozas, 2002), although Fu's test yields better results for large samples (Fu, 1997). Would the populations of *C. mydas* of the North Atlantic Basin be adjusted by the use of the sudden expansion model? If the molecular marker is selectively neutral, we should expect that equilibrated populations are not under selection or that the evolutionary forces are offset. For this reason, if migration existed it would not have significant effects on the distribution of gene frequencies; *i.e.*, a quasi-closed source population. The natural history of sea turtles, supported by our phylogenetic and phylogeographic results, is based on colonization and recolonizations. Consequently, the trace(s) of the demographic fluctuations could be deleted, as has happened in some human populations (Excoffier & Schneider, 1999). Our  $F_{ST}$  results indicated that the differentiation between the populations was due to the genes' distribution as opposed to the gene changes within the nesting population. Therefore, migration was the principal evolutionary force that drew the genes in the nesting population. This evolutionary force was demonstrated by historical failures to return to the natal site for breeding. During a remigration interval, a female can travel thousands of kilometers (Luschi *et al.*, 2003). If this female failed to return to her nesting site, she would contribute genetically to any one of the available rookeries within her migratory route. However, the rookeries have significant levels of endemism and are adjusted to the stepping stones model. This evidence demonstrates that the philopatry failures do not take place randomly. These failures could be considered a consequence of individual survival more than due to inherent failures of the species' natal homing behavior. Nevertheless, philopatry failures occurred; *e.g.*, the clinal variation showed by the haplotype CM-A5 whose frequency was increased with the decrease in latitude (as observed by Encalada *et al.* 1996). With the above in mind, we do not expect that the sudden expansion model could adjust for these nesting populations under the following demographic scenarios: I) a strong founder event of the best-distributed and best-represented haplotypes in each of the lineages; II) a redistribution of individuals as a consequence of drastic climatic variation during the Pleistocene; III) a population crash suffered as a consequence



of European colonization; and IV) a combination of all the above scenarios.

### Phylogenetic and Phylogeographic relationships

The choice of haplotype CM-A1 as the ancestor conflicts with the fact that haplotype CM-A3 is the best-distributed and best-represented in the nesting populations of the Greater Caribbean. The significant higher number of change existing among external haplotypes and CM-A1, and the intermediate position of CM-A1 regarding the other two diversification centers was taken into account for the identification as an ancestral haplotype.

In our analysis, the recent divergence of haplotypes from the nesting populations of the Western Greater Caribbean during the Superior Pleistocene corresponds with the few mutations that separate most of the haplotypes from the ancestral haplotype. This characteristic is shared by the Lineage I (Formia *et al.*, 2006). Nevertheless, this divergence happened before the separation of the present nesting populations. These nesting populations share, with more representativeness, the best-distributed haplotypes in the lineage (ancestor included) as a consequence of I) historical dispersion events (colonization and recolonization) that founded the rookeries with the best-distributed and best-represented haplotypes of the region, and II) demographic fluctuations in established demes that facilitated the diversification and the fixation of haplotype variants recognized as a low representative endemism. Both conditions were the result of events that occurred during historical time. They resulted in the current composition and population structure that has a greater differentiation within than between nesting populations of the region as a consequence of restricted genetic flow due to isolation by distance. Of course, we do not preclude the dispersions in ecological time caused by philopatry failures, which worked against population endemism; nevertheless, these kinds of dispersion have been less frequent and slower. They are evident in clines (CM-A5) and through the low nucleotide diversity in the majority of the nesting populations.

The interpretation of the geographical association of haplotypes in the clade integrated for the Cyprus and Quintana Roo nesting populations revealed an ancient genetic relationship between the diversification center (CM-A13) and the other haplotypes from American nesting populations. Nevertheless, the diversification time of the

haplotypes of Cyprus was not different from the rest of the haplotypes of the lineage (unpublished data). This haplotype's age predates the time when there existed a suitable habitat in the Mediterranean Sea for *C. mydas* to nest (Encalada *et al.* 1996); *i.e.*, after the Würm glaciation. Consequently, the haplotype (CM-A13) must have originated in a nesting population from the Greater Caribbean. In fact, this haplotype was found in a foraging habitat of east-central Florida (Bagley, 2003). Accordingly, we can infer that the haplotype endemism of the Cyprus nesting population is due to the gene fixation in Mediterranean Sea. This can be explained by the possibility that haplotype CM-A13 is rare within the studied nesting populations, or uncommon in other nesting populations that have not been studied with molecular markers; a combination of these variants; or that it simply belonged to now-extinct rookeries. These possibilities could better explain the presence of endemic haplotypes in other populations from nesting areas with unstable habitats for reproductive success during the glaciations, than the alternative conception of a great ancestral population in Florida (Allard *et al.*, 1994).

The nesting habitat of *C. mydas* is restricted to tropical and subtropical areas. At the height of the glacial period, due to the advance of the continental ice layer and the consequent reduction of the sea level (approximately 100 m below the current level at that time according to Bowen (1978)), the foraging and nesting habitats for *C. mydas* were probably diminished. Climatic records of the Wisconsin and Würm glaciations (which began ca. 18,000 years ago, and are also known as the Last glaciation), indicate that at that time marine ice extended several degrees of latitude closer to the equator than present day levels (Gates, 1993).

Consequently, due to low temperatures and high aridity, suitable habitats for the nesting *C. mydas* did not exist at southern latitudes of the North America and Europe where the current rookeries of the Florida and Yucatan peninsulas, and Cyprus Island are found (Encalada *et al.*, 1996). The equatorial regions could have been effective refuges for the species during the glaciations, with subsequent colonizations towards the high latitudes during the interglacial periods (Encalada *et al.*, *op.cit.*). Consequently, these dispersions over long distances during the retreat of the glaciers allowed the re-colonization of the current nesting areas in the northwest Caribbean and Mediterranean Sea.

## Conservation impact

The rookeries of southwestern Cuba constitute a relict population, with high genetic endemism and a moderate representation of individuals with endemic haplotype. The unique genetic diversity of these rookeries is being threatened by human activities. These rookeries, like others that are of small population size, have operated as a genetic reserve during events that have caused the extirpation of large nesting populations (Lahanas *et al.* 1994). Now that the composition of southwestern Cuban nesting population is known, contributions from source populations can not only be estimated with greater accuracy, but the presence and degree of contribution by Cuban rookeries can be established for the first time. Consequently, our results contribute to knowledge of the niche, and the migratory routes of individuals identified with the endemic Cuban haplotypes. This newfound knowledge creates new opportunities for international scientific collaboration in the sustainable management of the species and its habitat.

Finally, our results also provide useful information for the creation of protected marine areas (MPAs) in Cuba, directed toward the conservation of this declining species. MPAs could be effective in the conservation and protection of sea turtles if, during the determination of the geographic limits, the niche of this species and the evolutionary potential of the rookeries within the genetic background of the metapopulation are considered. These considerations are essential for effectively designing MPAs (Bowen & Roman, 2005).

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