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Support for Natal Homing in Green Turtles from Mitochondrial DNA Sequences

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Mitochondrial DNA (mtDNA) sequences of the control region were obtained for the Costa Rica and Florida colonies of the green turtle (*Chelonia mydas*) to test the hypothesis that gravid females return to their natal beaches to lay their eggs. Analyses of intra- and intergroup variation of these sequences revealed that the two colonies are structured differentially along maternal lineages and that mtDNA diversity is unusually high in the Florida population. The former result supports the hypothesis of natal homing in green turtles. For the latter, two explanations are provided: (1) that the Florida colony is the product of admixture (immigration from multiple sources); or (2) that it is a remnant of a larger, ancestral population. The presence or absence of Florida haplotypes among other western Atlantic populations will provide a critical test of these alternate hypotheses.

THE natal homing hypothesis for the reproductive migration of sea turtles suggests that sexually mature animals return to breed and nest at the same rookery from which they had hatched (Carr, 1967). The hypothesis has been most extensively developed for green turtles (*Chelonia mydas*), in large part because of the extensive tagging and recapture project of nesting females at Tortuguero, Costa Rica, and elsewhere (Carr et al., 1978; Carr et al., 1982). Tagging studies indicate that females have a strong propensity for nest-site fidelity, returning repeatedly to the same nesting beach. However, female philopatry is not perfect; infrequent errors in nesting may provide the source of individuals for the founding of new colonies (Carr, 1967).

One prediction of the natal homing hypothesis is that each nesting colony should comprise a group of isolated maternal lineages as females assort themselves according to their natal beach. As such, mitochondrial DNA (mtDNA) differences are expected to accumulate among colonies, given the maternal inheritance of this genome (Avisé et al., 1987; Meylan et al., 1990; Avisé, 1991). Recently, the natal homing hypothesis was tested for 15 colonies of green turtles in the Pacific and Atlantic regions using restriction-fragment-length-polymorphisms (RFLP) of mtDNA (Bowen et al., 1992). This study identified significant differences among most colonies, thereby extending the earlier conclusions based on mtDNA analyses in favor of natal homing (Meylan et al., 1990).

However, certain geographically separated populations showed no significant mtDNA differences on the basis of restriction-site data. Because turtles as a group are characterized by slow rates of molecular evolution (Bowen et al., 1989, 1991; Avise et al., 1992), the absence of genetic divergence in these cases may be attributed to the overall paucity of available genetic markers rather than to a failure of the hypothesis. Indeed, Bowen et al. (1992) commented on the limited variation detected with RFLP analysis (i.e., 14 haplotypes in 226 individuals surveyed with an average of 106 restriction sites), even among those populations that were fixed for alternate mtDNA genotypes.

In this earlier study, some rookery pairs were fixed or nearly fixed for the same haplotype, and it could not be determined whether these results were the product of historical associations (recent coancestry) or regional gene flow. Two populations that exhibited no significant mtDNA differences were the colonies at Tortuguero, Costa Rica, and Hutchinson Island, Florida. Bowen et al. (1992) reported that the Costa Rica colony was fixed for one haplotype (designated type A), which was shared with the Florida population at a frequency of 0.88. In the Florida colony, a second haplotype (B) was detected at a frequency of 0.12. The two haplotypes differed by a single mutation detected with the restriction enzyme *Dra* I.

The rookery at Tortuguero is the largest one remaining in the Atlantic Ocean with an annual population of nesting females ranging from 5000 to 52,000 individuals (Carr et al., 1982). In contrast, the Florida rookery is relatively small, with approximately 400 breeding females (Witherington and Ehrhart, 1989). The purpose of this study is to determine whether these two colonies are distinct in terms of mtDNA genealogies as predicted under the natal homing hypothesis. To ensure that additional variation is surveyed, our study has focused on sequencing the 5'-end of the mtDNA control region. In other vertebrates, this segment evolves at least six to 10 times faster than the overall genome (Vigilant et al., 1991; Edwards, 1992; Quinn, 1992), thereby allowing for a more rigorous test of natal homing in these populations.

MATERIALS AND METHODS

The mtDNA samples for the Costa Rica ($n = 15$) and Florida ($n = 24$) rookeries were the same specimens used by Bowen et al. (1992) and Karl et al. (1992) to analyze RFLP variation in the mitochondrial and nuclear DNAs of green turtles. Initially, primers L16007 (Kocher et al.,

1989) and HD3 [5' AAGAGCAAGTTCATG-GGAATCGGCAAGT 3', for the heavy strand (MMM, MWA, and J. M. Moreno, unpubl.)] were employed to amplify and sequence the 5'-end of the control region for single representatives of each rookery. In these experiments, the polymerase chain reaction (PCR) and sequencing protocols of Allard et al. (1991) were followed, relying on the ds-DNA Cycle Sequencing System of Bethesda Research Laboratories. Turtle-specific primers (LTCM1, LDCM1, and HDCM1; Appendix) were then developed from this information. The remaining 37 samples were amplified and sequenced with the new primers. In these experiments, three polymorphic sites (see Appendix) were identified and targeted for all individuals. For all 39 samples, standard precautions were taken to avoid contamination (Erlich et al., 1991). Negative controls (PCR reactions without any DNA) were routinely conducted to detect the presence of foreign DNAs.

Confirmation of these orthologues as from the control region and/or the adjacent tRNA^{PRO} gene was obtained by aligning the sequences for the original representatives of each rookery to the known mtDNA genomes of other higher vertebrates [e.g., domestic chicken (Desjardins and Morais, 1990) and harbor seal (Arnason and Johnsson, 1992)]. An overall alignment for green turtle sequences was done by eye because only three polymorphic sites and no length variation were observed.

Within-population variation was quantified for each colony as haplotype diversity (h_{CR} and h_{FL}) and nucleotide diversity (π_{CR} and π_{FL}), where the subscripts refer to Costa Rica and Florida, respectively; Nei, 1987: equations 8.5 and 10.6). For haplotype diversity, standard errors were estimated as the square root of their sampling variances (Nei, 1987: formula 8.12), after modification of the equation for haploid populations. Between populations, haplotype frequencies were compared with the G -test of independence (Sokal and Rohlf, 1981). Sequence differences between the two colonies were quantified as total and net divergences [$d_{CR-FL(TOT)}$ and $d_{CR-FL(NET)}$, respectively; Nei, 1987: equations 10.20 and 10.21]. Estimates of migration between the two populations (Nm) were calculated by the G_{ST} approach [with G_{ST} estimated according to Nei (1987: p. 191) and $Nm = \{0.5(1/G_{ST} - 1)/[(L - 1)/L]^2\}$, where L equals the number of populations (Takahata and Palumbi, 1985)] and by the private alleles method (Barton and Slatkin, 1986; Slatkin and Barton, 1989).

Only the nucleotide data for the noncoding

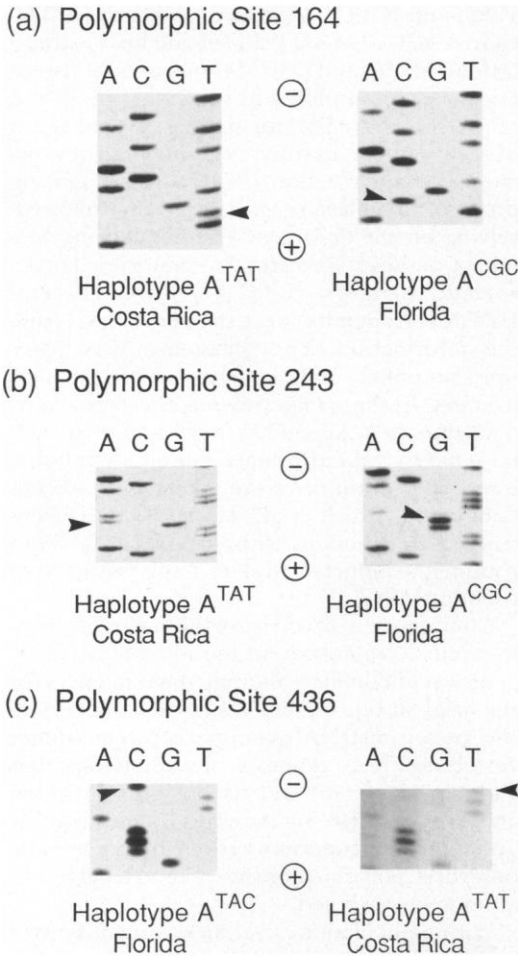


Fig. 1. Representative autoradiographs illustrating the three sequence polymorphisms of the control region in green turtles. Arrowheads highlight the specific polymorphic sites.

control region (sites 72–620; Appendix) were used in the calculations of $\hat{\pi}$ and d to permit more accurate estimates of sequence variation in this part of the mtDNA molecule. For the same reasons, these calculations did not include the restriction-site data because much of this information represents coding regions of mtDNA. However, the available data for the control region were maximized by counting all noncoding positions per pair rather than limiting each comparison to only those sites [76–444 (Appendix)] common to all 39 individuals.

RESULTS

A total of 17,909 base positions (bp) were obtained for the 39 samples of green turtles, with 99% of this information from the 5'-end

TABLE 1. FREQUENCIES OF mtDNA HAPLOTYPES IN THE COSTA RICA AND FLORIDA COLONIES OF GREEN TURTLES. The base-letter for each haplotype designation corresponds to the A- or B-restriction profile of Bowen et al. (1992), whereas the superscripts refer to its nucleotides at polymorphic positions 164, 243, and 436, respectively (Fig. 1). For example, haplotype A^{TAC} has restriction pattern A (following Bowen et al., 1992) and nucleotide T at polymorphic site 164, A at 243, and C at 436.

mtDNA haplotype	Colony	
	Costa Rica	Florida
1. A ^{TAC}	14	12
2. A ^{CAC}	0	8
3. A ^{CGC}	0	1
4. B ^{CAC}	0	3
5. A ^{TAT}	1	0
Totals	15	24

of the control region (Appendix). On average, the Costa Rica and Florida populations were scored for 470 bp and 452 bp per individual, respectively, with an overall mean of 459 bp per turtle. Eleven Florida and 10 Costa Rica samples were sequenced for over 95% of the 510 bp-region spanning primers LTCM1 and HDCM1 (Appendix). This included one individual from Florida with the B-haplotype (Bowen et al., 1992).

Sequence polymorphisms representing transitions were detected at three positions in the control region (sites 164, 243, and 436) (Appendix; Fig. 1). None of these differences was located in a *Dra* I site, and, as such, the restriction site change that distinguishes haplotypes A and B in the Florida population (Bowen et al., 1992) is separate from the observed variation in the control region. The restriction-site results can, therefore, be combined with the control region sequence data to distinguish five distinct haplotypes (Table 1), which are interrelated by single substitutions as shown in Figure 2.

Four haplotypes at frequencies of 0.50 (A^{TAC}), 0.33 (A^{CAC}), 0.13 (B^{CAC}), and 0.04 (A^{CGC}) were detected in the Florida colony (see Table 1 for a description of our haplotype notation). In contrast, only two haplotypes were observed in the Costa Rica population (A^{TAC} and A^{TAT} at frequencies of 0.93 and 0.07, respectively). Only the A^{TAC} haplotype was, therefore, shared by the two populations. Haplotype and nucleotide diversities for the Costa Rica colony were 0.13 (95% confidence interval of 0.00–0.35) and 0.03%, respectively. For the Florida population, these estimates were 0.65 (0.52–0.78) and 0.15%.

The haplotype frequencies of the two populations were significantly different ($G = 11.08$ with the B^{CAC} , A^{CGC} , and A^{TAT} classes combined; $df = 2$; $P < 0.005$). Total divergence between the two [$d_{CR-FL(TOT)} = 0.14\%$] was slightly less than the nucleotide diversity for Florida alone ($\hat{\pi}_{FL} = 0.15\%$). After correcting for average nucleotide diversity, net divergence was estimated as 0.06% . The G_{ST} estimate of migration was 0.7 migrants per generation (with $G_{ST} = 0.15$), which approximates that of 0.9 migrants per generation for the private alleles method.

DISCUSSION

Combining the mtDNA sequence data for the control region with the restriction-site information (Bowen et al., 1992) offers the greatest resolution for comparing the Costa Rica and Florida populations of green turtles. When taken together, the number of haplotypes increases from two (as reported by Bowen et al., 1992) to five (Table 1). Based on the restriction-site data, the Costa Rica and Florida populations are characterized by no variation vs two haplotypes, \hat{h}_{FL} of 0.22 , and $\hat{\pi}_{FL}$ of 0.02% , respectively. In contrast, these estimates become two haplotypes and \hat{h}_{CR} of 0.13 vs four haplotypes and \hat{h}_{FL} of 0.65 when the two data sets are combined. Similarly, $\hat{\pi}_{CR}$ and $\hat{\pi}_{FL}$ become 0.03% and 0.15% , respectively, with the control region sequences. Relative to the restriction-site information, resolution is thereby enhanced by a factor of 7.5 according to the $\hat{\pi}_{FL}$ values (i.e., 0.15% vs 0.02% , respectively).

With this additional resolution, significant differences are found between the Costa Rica and Florida colonies. The haplotype frequencies of the two populations become significantly different (G -test; $P < 0.005$). Both estimates of migration (Nm) indicate that the two colonies share approximately 0.7 – 0.9 migrants per generation. Given that mtDNA is effectively a single locus, these values of Nm must be interpreted with caution. Nevertheless, both values suggest a reduced level of gene exchange, whereby migration is insufficient to offset the diverging effects of genetic drift (Slatkin and Barton, 1989). Alternatively, such estimates may reflect the retention of shared haplotypes from a common ancestor rather than an ongoing low level of migration. In either case, the mtDNA results indicate that the two populations are structured differentially along maternal lines, thereby providing further support for the natal homing hypothesis in green turtles. The inability of earlier studies to distinguish these two colonies with restriction-site data is thereby at-

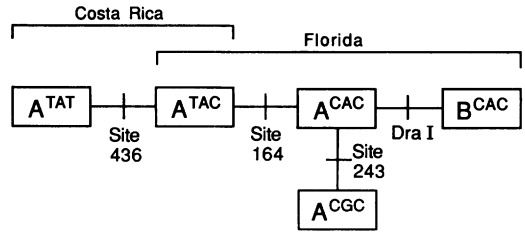


Fig. 2. Minimum-length, unrooted tree for the five mtDNA haplotypes observed in the Costa Rica and Florida colonies. Sequence changes among haplotypes are represented by cross-marks corresponding to the three polymorphic positions of the control region (Appendix; Fig. 1) and the *Dra* I difference reported by Bowen et al. (1992).

tributed to a lack of resolving power, rather than to ongoing gene flow.

Integrating the sequence data for the control region with the restriction-site information further reveals that the Florida colony is five times more polymorphic than the Costa Rica population. The difference between \hat{h}_{CR} and \hat{h}_{FL} (0.13 vs 0.65 , respectively) is significant (t-test; $t = 3.98$; $P < 0.001$), a result supported as well by estimates of nucleotide diversity ($\hat{\pi}_{CR} = 0.03\%$, $\hat{\pi}_{FL} = 0.15\%$). Slightly more sequence variation exists within the Florida population (0.15%) than between the two colonies ($d_{CR-FL(TOT)} = 0.14\%$) suggesting that the common ancestor of the Costa Rica and Florida rookeries was relatively recent. These results are the opposite of those predicted by neutrality theory and current rookery-population sizes (see below).

The hypothesis that the mtDNA variation in the Florida sample is unusually high for green turtles (rather than atypically low for the Costa Rica colony) is supported by the restriction-site data (Bowen et al., 1992). Out of 15 populations, eight showed no variation (including the Costa Rica sample), whereas only three (Hawaii, Japan, and Polynesia) exhibited \hat{h} and $\hat{\pi}$ values both greater than those for Florida (0.22 and 0.02% , respectively). Furthermore, turtles overall (including *C. mydas*) are characterized by unusually low rates of mtDNA evolution and diversity, compared to other vertebrates (Avisé et al., 1992). Thus, an explanation for the significant differences in variation between these colonies must focus on the Florida population to understand why its diversity is so great.

An explanation for the increased diversity in the Florida sample must also take into account that this rookery currently consists of 350 – 400 nesting females and may never have been much larger in historical times (Dodds, 1982; Witherington and Ehrhart, 1989). This population

also may be relatively new to Florida (i.e., less than 10,000 years old; see comments in Bowen et al., 1992). Thus, the predicted mtDNA diversity in this population should be low, not high as revealed in this study (Wilson et al., 1985; Avise et al., 1988). One possibility is that mutation rates of mtDNA are also high relative to other colonies (as represented here by the Costa Rica sample). However, this explanation is considered unlikely because turtles as a group (including *C. mydas*) show similar, slow rates of mtDNA change relative to other vertebrate taxa (Avise et al., 1992).

A more likely hypothesis is that the Florida colony contains females derived from other populations in the western Atlantic. In short, this hypothesis maintains that the increased mtDNA diversity in the Florida colony is the result of admixture (recent and/or ongoing immigration to this population from different sources). Perhaps the east coast of Florida is a focal point for colonization, because of ocean currents, coastal topography, or other geological and oceanographic conditions. Low levels of migration to sites, other than their natal beach, form part of the natal homing hypothesis because such accidents are viewed as essential for the founding of new colonies in a changing environment (Carr, 1967; see also below). By receiving immigrants from multiple sources, increased mtDNA diversity is expected in the Florida colony even though the number of nesting females remains small.

The hypothesis of admixture in the Florida colony depends on the assumption that current nesting colonies are indicative of long-term population sizes for the Costa Rica and Florida rookeries. Given that this assumption cannot be substantiated by current evidence, an alternate hypothesis must be considered to explain the greater mtDNA diversity in the Florida rookery relative to the Costa Rica population. This explanation maintains that the Florida rookery is derived from a larger, ancestral population. According to this hypothesis, present levels of mtDNA diversity reflect a historically large population in the vicinity of the current rookery on the Florida peninsula (see Avise et al., 1988). In support of this hypothesis, we note that large rookeries existed in historical times at nearby locations in the Caribbean, including Grand Cayman Island and the Dry Tortugas (Parsons, 1962).

The two hypotheses, therefore, provide alternate explanations for the origin of the greater mtDNA diversity in the Florida colony. The admixture hypothesis argues for multiple sources for the Florida female lineages, whereas the alternate explanation maintains that the

higher mtDNA diversity is the product of a larger, ancestral colony in Florida. The former, therefore, predicts that the "unique" Florida haplotypes (Table 1) will be shared with other colonies in the western Atlantic, a condition that does not apply to the alternate possibility. Thus, one test of the admixture hypothesis is to survey other western Atlantic colonies for the Florida haplotypes. Samples from nesting populations near Florida (e.g., from the Bahamas) will be of particular importance in these tests, as will those from the Yucatan Peninsula, given its closer geographic proximity in the Pleistocene during the most recent glacial maximum (Avise, 1992). Failure to find the "unique" Florida haplotypes elsewhere will provide evidence against the admixture hypothesis, thereby favoring the explanation of a larger, ancestral population.

During the mid-1960s and 1970s, green turtles from Costa Rica were released at several sites in Florida as hatchlings and yearlings (Carr and Sweat, 1969; Carr, 1979). However, their impact on the conclusions of this study is considered small for two reasons. First, if green turtles do imprint on the beach to which they return to breed, the appropriate time period for imprinting (i.e., possibly within 24 hours after hatching) had almost certainly elapsed before hatchlings (most of which were held in Costa Rica for at least several days before being transported) and yearlings were released on Florida beaches (Carr, 1979). Second, because growth rates of green turtles are very slow (Bjorndal and Bolten, 1988), if any released individuals survived, it is unlikely that they would have reached sexual maturity by 1987, when the samples for this study were collected. In any case, the natal homing and admixture hypotheses do not depend on whether the released individuals have had a minimal impact on the current results. The Costa Rica and Florida populations are significantly different in mtDNA frequencies, despite any dampening effects of this human-mediated migration. Thus, a stronger case for the natal homing hypothesis is admitted for the current mtDNA results if the impact of the released individuals is greater than suggested above. In an analogous way, the admixture hypothesis does not preclude the possibility of immigration by human intervention. Rather, its primary argument is that the increased diversity in the Florida population is the result of immigration, whether by natural or human-assisted means. Thus, neither of these explanations relies on an assumption that the released turtles have had a small impact on the Florida colony.

Conclusions in this report regarding the natal

homing hypothesis and the greater diversity in the Florida colony only apply to the female components of population structure. Indeed, a more complete picture of the life history and population genetics of green turtles has been suggested by Karl et al. (1992) using an RFLP analysis of nuclear DNA (with a biparental vs maternal mode of inheritance). This investigation revealed a lower level of nuclear DNA structuring among populations relative to the mtDNA results, leading to a hypothesis of moderate male-mediated gene flow among colonies. Taken together, the mtDNA and nuclear DNA results support the existence of distinct maternal and paternal components of population structure in green turtles (Bowen et al., 1992; Karl et al., 1992).

Unlike most other populations of sea turtles, the Florida colony of *C. mydas* is apparently increasing (Dodd, 1982; Conley and Hoffman, 1987). Nevertheless, it remains on the U.S. List of Endangered Species. This study indicates that the Florida population is genetically distinct and characterized by unusually high mtDNA diversity. Because the goal of conservation programs is to maintain diversity, the protected status of this rookery is well deserved.

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APPENDIX. MITOCHONDRIAL DNA SEQUENCES OF THE 5'-END OF THE CONTROL REGION AND ADJACENT tRNA^{PRO} GENE. The two are shown in uppercase (sites 72–620) and lowercase (positions 1–71) lettering, respectively. At polymorphic sites 164, 243, and 436, the nucleotides for haplotype A^{TAC} are presented with the rest of the sequence, with the alternate bases for the other haplotypes shown in parentheses. Dashes mark the locations of annealing for the new oligonucleotide primers used in PCR amplification and sequencing [(LTCM1) 5' CCCAAAACCGGAATCCTAT 3'; (LDCM1) 5' AGTGAAATGACATAGGACATA 3'; and (HDCM1) 5' ACTACCGTATGCCAGGTTA 3']. The sequence for haplotype A^{TAC} has been deposited in the GenBank database under accession number M98394.

tcaaaagagaaggacttaaaccttcatccccgggtcccaaaaccggaatcctataattaactatccttt 70
 ----- (LTCM1)

gACACAGGAATAAAAGTGTCACACAAACTAACTACCTAAATTCTCTGCCGTGCCCAACAGAACAATACC 140

CGCAATACCTATCTATGTATTATTGTACATCTACTTATTTACCAATAGCATATGACCAGTAATGTTAACA 210
 (C)

GTTGATTTGGCCCTAAACATAAAAAATCATTGAATTTACATAAATATTTTAAACACATGAATATTAAGCA 280
 (G)

GAGGATTAAGTGAATGACATAGGACATAAAATTAACTATTATACTCAACCATGAATATCGTCACAG 350
 ----- (LDCM1)

TAATTGGTTATTTCCCTAAATAGCTATTCACGAGAAATAAGCAACCCTTGTTAGTAAGATACAACATTACC 420

AGTTTCAAGCCCATTCAGTCTGTGGCGTACATAAATTTGATCTATTCTGGCCTCTGGTTAGTTTTTCAGGC 490
 (T)

ACATACAAGTAACGACGTTTCATTCGTTCCCTTTAAAGGCCTTTGGTTGAATGAGTTCTATACATTAAA 560

TTTATAACCTGGCATAACGGTAGTTTTACTTGCATATAGTAGTTTTTTTTCTCTCTGTGTT 630
 ----- (HDCM1)
