Global Population Genetic Structure and Male-Mediated Gene Flow in the Green Turtle (*Chelonia mydas*): RFLP Analyses of Anonymous Nuclear Loci

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ABSTRACT

We introduce an approach for the analysis of Mendelian polymorphisms in nuclear DNA (nDNA), using restriction fragment patterns from anonymous single-copy regions amplified by the polymerase chain reaction, and apply this method to the elucidation of population structure and gene flow in the endangered green turtle, *Chelonia mydas*. Seven anonymous clones isolated from a total cell DNA library were sequenced to generate primers for the amplification of nDNA fragments. Nine individuals were screened for restriction site polymorphisms at these seven loci, using 40 endonucleases. Two loci were monomorphic, while the remainder exhibited a total of nine polymorphic restriction sites and three size variants (reflecting 600-base pair (bp) and 20-bp deletions and a 20-bp insertion). A total of 256 turtle specimens from 15 nesting populations worldwide were then scored for these polymorphisms. Genotypic proportions within populations were in accord with Hardy-Weinberg expectations. Strong linkage disequilibrium observed among polymorphic sites within loci enabled multisite haplotype assignments. Estimates of the standardized variance in haplotype frequency among global collections ($F_{ST} = 0.17$), within the Atlantic-Mediterranean ($F_{ST} = 0.13$), and within the Indian-Pacific ($F_{ST} = 0.13$), revealed a moderate degree of population substructure. Although a previous study concluded that nesting populations appear to be highly structured with respect to female (mitochondrial DNA) lineages, estimates of Nm based on nDNA data from this study indicate moderate rates of male-mediated gene flow. A positive relationship between genetic similarity and geographic proximity suggests historical connections and/or contemporary gene flow between particular rookery populations, likely via matings on overlapping feeding grounds, migration corridors or nonnatal rookeries.

MOLECULAR techniques have revolutionized studies of genetic variation and gene flow in natural populations. Protein electrophoretic techniques are widely employed for such purposes, but the limited number of detectable allozyme polymorphisms in some taxa has prompted searches for additional sources of molecular markers. Restriction site analyses of mitochondrial DNA (mtDNA) typically uncover extensive variability, but inferences about population structure are confined to female lineages.

More recently, studies of restriction fragment length polymorphisms (RFLPs) in nuclear DNA (nDNA) have expanded molecular data bases by revealing additional Mendelian variation at numerous independent and biparentally inherited loci. To date, most such studies have involved Southern blot analyses (BHATTACHARYA and DRUEHL 1989; NEVO, BEN-SHLOMO and MAEDA 1989; SIMMONS *et al.* 1989) and have found applications in genome and quantitative trait loci mapping (BOTSTEIN *et al.* 1980; MARTIN *et al.* 1989; PATERSON *et al.* 1988; WELLER, SOBER and BRODY 1988), breeding studies and strain verification (APUYA *et al.* 1988; BECKMANN *et al.* 1986; CASTLE, HORGEN and ANDERSON 1987; MCDERMOTT *et al.* 1989), analyses of hybridization and parentage (HALL 1990; KEIM *et al.* 1989; QUINN *et al.* 1987), and assessment of genetic relationships among species or higher taxa (ANDERSON, PETSCHE and SMITH 1987; LOUKAS, DELIDAKIS and KAFATOS 1986). Few studies have used RFLP approaches to examine questions of geographic population genetic structure and gene flow [but see QUINN and WHITE (1987)], or utilized the polymerase chain reaction (PCR) in the detection of RFLP polymorphisms (but see HORN, RICHARDS and KLINGER (1989) and ROWAN and POWERS (1991)].

This study introduces an approach for rapid screening of Mendelian RFLPs from anonymous single-copy nDNA that takes advantage of PCR and avoids the labor intensive and relatively expensive technique of Southern blotting. We apply this approach to an analysis of population genetic structure and gene flow in the green turtle *Chelonia mydas*, an endangered marine turtle that is globally distributed in tropical and subtropical waters. The motivations for this particular

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application are twofold. First, a previous protein electrophoretic survey of green turtles failed to reveal significant differentiation between scattered nesting rookeries around the world (BONHOMME *et al.* 1987), a result that the authors attributed to extensive gene flow. However, this conclusion should be viewed with caution because only two loci (of 23 assayed) were polymorphic, and these polymorphisms occurred at a single locale. SMITH *et al.* (1977) described higher levels of allozyme variation in the green turtle, but they analyzed only two Atlantic populations in close geographic proximity and one of these was the source of the polymorphisms found by BONHOMME *et al.* (1987). Second, a recent analysis of mtDNA revealed that many green turtle nesting populations are distinguishable by fixed or nearly fixed haplotype differences, indicating severe limitations to gene flow between rookery populations (BOWEN *et al.* 1992). However, this conclusion applies only to the female lineages and therefore does not address unresolved questions concerning male-mediated gene flow or the mating system.

Results from physical tag returns have shown that female green turtles often migrate great distances (sometimes thousands of kilometers) from foraging grounds to nesting beaches and that each adult female normally returns to the same locale to lay eggs throughout her adult life (CARR 1986; CARR, CARR and MEYLAN 1978; MEYLAN 1982). With respect to maternally inherited mtDNA lineages, *C. mydas* exhibits an extensive phylogeographic structure (BOWEN, MEYLAN and AVISE 1989; BOWEN *et al.* 1992; MEYLAN, BOWEN and AVISE 1990). A deep division in the global mtDNA phylogeny between Atlantic-Mediterranean *vs.* Indian-Pacific samples evidences a historical population separation mediated by continental barriers. A shallower but pronounced genetic differentiation among rookeries within ocean basins indicates a strong behavioral predisposition for natal homing by females.

In contrast, since males seldom come ashore (where they might otherwise be observed and tagged), little is known about male dispersal and migratory behavior, nor about the green turtle mating system. Scattered observations of mating behavior have been made on feeding grounds (ROSS 1984), during migration (MEYLAN, MEYLAN and YEOMENS 1991), adjacent to nesting beaches (BOOTH and PETERS 1972; DIZON and BALAZS 1982), and in spatially distinct breeding areas. At least some males accompany females to nesting areas, and there is some evidence for male philopatry to breeding site (DIZON and BALAZS 1982). Nonetheless, the mating system and the extent of male-mediated gene flow between rookeries, and its population genetic consequences, have remained almost totally unexplored.

Here we employ nuclear RFLP analyses to describe 12 polymorphisms in green turtles from the Atlantic, Mediterranean, Indian and Pacific Ocean basins. The goals of this study were to (1) develop a new, PCR-based approach for the generation of nuclear, Mendelian RFLPs, and (2) apply the results to unresolved questions concerning the green turtle mating system and migratory behavior. We are particularly interested in the pattern of nuclear population genetic structure on a global scale, the mating structure within ocean basins, and the magnitude of male-mediated gene flow between rookeries, especially those known to be differentiated with respect to mtDNA lineages.

MATERIALS AND METHODS

Selection of loci: Locus specific primers used in the PCR amplifications were made from single- or low-copy-number clones isolated from a total cell DNA library. Total cell DNA was isolated from the soft tissue of a single green turtle hatchling by a procedure modified from HERRMANN and FRISCHAUF (1987). Muscle, liver, kidney and heart tissues were ground in 100 mM Tris-HCl, pH 8.0, 100 mM NaCl and 100 mM EDTA at room temperature. The homogenate was lysed by the addition of sodium dodecyl sulfate and proteinase K, and the lysate was extracted with phenol and chloroform. The DNA was precipitated with the addition of sodium acetate and ethanol. The library was constructed by digesting approximately 30 μg of total cell DNA to completion with the restriction endonuclease *Hin*dIII. Fragments in the size range of 500 to 5000 base pair (bp) were isolated by gel electrophoresis onto DE-81 paper (Whatman International, Ltd., Maidstone, England) and cloned into the phagemid vector Bluescript SK plus (Stratagene Cloning Systems).

Recombinant phagemids were recovered by transforming *Escherichia coli* JM101 cells and plating under selection conditions. Putative recombinants were screened for inserts by *Hin*dIII digestion of mini-DNA preparations (MANIATIS, FRITSCH and SAMBROOK 1982) and genomic copy number was determined by dot blot analysis using labeled total cell DNA as a probe. Since the radioactive signal intensity of the clone dot is an indication of its genomic copy number, signals were classified as (a) absent, (b) weak, (c) moderate or (d) strong, corresponding to single, low, moderate and high genome copy number, respectively. Single- and low-copy clones with inserts between 500 and 2000 bp were selected for further analysis. The sequences of the first 100–150 bp from both ends of these clones were determined by the dideoxy chain termination method using a Sequenase T_7 sequencing kit (U.S. Biochemical) according to the manufacturer's recommendations. Each clone was designated as pdCM (p = plasmid; d = DNA source; CM = *Chelonia mydas*) and numbered. The sequences of potential PCR primers were selected using the computer program NAR (RYCHLIK and RHOADS 1989) such that primers were approximately 20 nucleotides long, had an intermediate duplex stability ($155 < \Delta H^\circ < 165$), little or no secondary structure and small (less than four nucleotides) or no interprimer complementarity. In addition, the sequences were analyzed for putative open reading frames using the MAP routine in the GCG sequence analysis software package (ver. 7; DEVEREUX, HAEBERLI and SMITHIES 1984). If an open reading frame was found, the 3′ end of the primer was located at the second nucleotide position of a putative codon. Primers

were chemically synthesized by Oligo's Etc. (Gilford, Connecticut). Each primer pair was designated as CM and numbered corresponding to the genomic clone from which it was derived.

Genotype determination: Total cell DNA was purified from specimens collected originally for mtDNA analyses (BOWEN *et al.* 1992). For some of the samples, tissue was unavailable, so nDNA was obtained from the linear fraction generated by the cesium chloride (CsCl) gradient isolation of mtDNA. The linear fraction is routinely collected during mtDNA studies in our lab, and is generally stored in the dark at 4°, without removal of the ethidium bromide (EtBr) or CsCl. For use in PCR amplification, the EtBr was removed by 1-butanol extraction and the DNA was separated from the CsCl by precipitation. Fresh frozen tissue samples were available from the remaining individuals and DNA was isolated from these samples as described in the section above. All DNA was stored in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer at 4°.

To identify polymorphic restriction sites, DNA from nine individuals (three from each of three geographically remote regions) was amplified with the anonymous primers and *Taq* polymerase (Promega) under the manufacturer's standard reaction conditions with the addition of $MgCl_2$ and bovine serum albumin to a final concentration of 2.5 mM and 0.1 μg/μl, respectively. Thermal-cycling parameters varied depending on the primers used. However, the only commonly changed condition was the annealing temperature (which varied from 54° to 62°). After amplification, 10 μl of each sample was electrophoresed in a 1.2% agarose gel to check the efficiency and fidelity of amplification. From each successful amplification, 10 μl of DNA were digested without purification. Restriction fragment patterns for 40 restriction endonucleases (*Alu*I, *Ava*I, *Ava*II, *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Bst*NI, *Cfo*I, *Cla*I, *Dde*I, *Dra*I, *Dra*II, *Eco*RI, *Eco*RV, *Fok*I, *Hae*III, *Hin*dII, *Hin*fI, *Kpn*I, *Ksp*I, *Msp*I, *Mvn*I, *Nci*I, *Nde*I, *Nde*II, *Nsi*I, *Pst*I, *Pvu*I, *Pvu*II, *Rsa*I, *Sac*I, *Sal*I, *Sma*I, *Spe*I, *Stu*I, *Taq*I, *Xba*I and *Xho*I) were determined by electrophoresis in a 2.5% agarose gel stained with 250 ng/ml ethidium bromide. Bands were visualized under short-wave ultraviolet light.

Individual polymorphisms were designated "+" or "−" for the presence or absence of a restriction site, respectively. For loci with more than one polymorphic site, the composite genotype was designated as *XX/XX* with the first "*X*" referring to the first polymorphic site on one of the chromosomes, the second "*X*" referring to the second site on the same chromosome (*cis* configuration), the "/" separating the two chromosome designations, and the other chromosome represented similarly after the slash. In the actual genotype assignment, each "*X*" is replaced with "+" or "−" to indicate the condition of the polymorphic sites. For example, an individual homozygous for the presence of one site and heterozygous for the presence of a second site would be designated + +/+ −.

Green turtle specimens: For the polymorphic systems, we assayed 256 individual nonsibling hatchlings collected from 15 locations worldwide (Figure 1). These include the 226 specimens from 15 locales presented in BOWEN *et al.* (1992). The specimens in the current survey from Quintana Roo, Mexico (MEX-A) were too degraded to be used in the mtDNA survey, but provided more than adequate templates for PCR analysis.

Statistical analyses: Population genetic analyses were performed using the BIOSYS-1 computer package (release 1.7; SWOFFORD and SELANDER 1981). Estimates of interrookery gene flow (*Nm*) were derived from F_{ST} values (WRIGHT 1951) as discussed by SLATKIN (1985) and STATKIN and BARTON (1989), using the relationship $Nm = ((1/F_{ST}) - 1)/4$. Statistical significance of F_{ST} values was assessed by χ^2 analysis following WORKMAN and NISWANDER (1970). *G*-tests, using Yates' correction for small sample size where appropriate, were employed to assess the significance of haplotype frequency differences among locales (SNEATH and SOKAL 1973). Phenetic relationships among rookery populations were summarized by UPGMA analysis (SNEATH and SOKAL 1973) of genetic distances (NEI's (1978) unbiased *D* values).

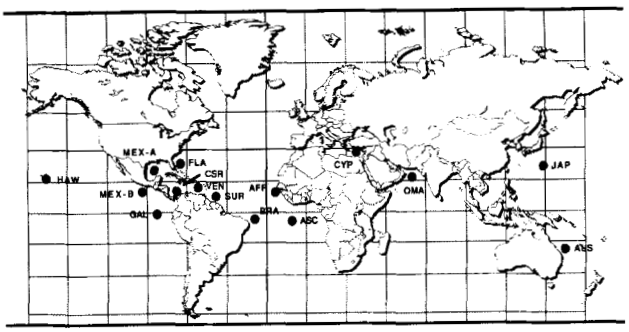

FIGURE 1.—Green turtle collecting sites and sample sizes. Abbreviations for collection sites and sample sizes are as follows: HAW, French Frigate Shoals, Hawaii, United States (N = 22); MEX-B, Michoacan, Mexico (7); GAL, Isabela Island, Galapagos, Ecuador (8); FLA, Hutchinson Island, Florida, United States (23); MEX-A, Quintana Roo, Mexico (7); CSR, Tortuguero, Costa Rica (23); VEN, Aves Island, Venezuela (9); SUR, Matapica, Suriname (15); ASC, Ascension Island, United Kingdom (47); BRA, Atol das Rocas, Brazil (17); AFR, Guinea Bissau, Africa (19); CYP, Lara Bay, Akamas Peninsula, Cyprus (9); OMA, Ras Al Had, Oman (15); JAP, Bonin Islands, Ogasawara Archipelago, Japan (19); AUS, Heron Island, Queensland, Australia (16).

RESULTS

Preliminary screen for RFLP variation: Seventy genomic clones were arbitrarily chosen, colony purified and assayed for insert size and copy number. The average size of an insert was approximately 1500 bp. As determined by dot blot analysis, 29 clones (41%) represented single copy sequences, 17 (24%) were low copy, 13 (19%) were moderate copy and 11 (16%) were high copy. Fifteen of the single or low copy clones with insert sizes ranging from 930 bp to 1380 bp were chosen and primers were made as described above. Of these 15 primer pairs, seven successfully amplified nDNA and produced a single amplification product of the expected size. The sequences of these primers are provided in the APPENDIX.

In the preliminary screens for RFLPs, small numbers of green turtles were analyzed using these seven primers (Table 1). The data presented were corrected for overlap of the recognition sequences of some of the enzymes. For example, *Taq*I recognizes the sequence 5′-TCGA-3′ which is a subset of the sequence 5′-GTCGAC-3′ recognized by *Sal*I. In this case, the "total number of sites observed" (Table 1) was counted simply as the number of *Taq*I sites, and the "total number of base pairs surveyed" was calculated as (4 ×

TABLE 1

Summary of the preliminary screen for RFLPs at seven nuclear loci

Primer	Fragment size (bp)	No. of cutting enzymes	No. of noncutting enzymes	Total No. of sites observed	Total No. of bp surveyed[a]	Percent sequence surveyed	Percent bp polymorphic[b]	No. sites polymorphic
CM-01	1380	17	21	26	115	8.3	0.00	0
CM-12	1195	20	20	29	140	11.7	1.43	2
CM-14	930	15	25	19	79	8.5	2.53	2
CM-28	1400	22	18	33	157	11.2	0.00	0
CM-39	1350	13	27	23	106	7.9	0.94	1
CM-45	1000	10	30	14	64	6.4	1.56	1
CM-67	1160	12	28	22	97	8.4	3.09	3
Overall	8415	15.6[c]	24.1[c]	166	758	9.0[c]	1.19[c]	9

Nine individuals (18 haplotypes) from three geographically remote regions were surveyed with up to 40 enzymes to detect polymorphic restriction sites (see MATERIALS AND METHODS).

[a] Total number of base pairs calculated as the number of observed restriction sites for an enzyme times the number of base pairs in the recognition sequence (NEI 1987), summed across all enzymes.

[b] Calculated as number of polymorphic sites divided by the total number of bp surveyed, with the assumption that each polymorphic site represents a single nucleotide difference.

[c] Numbers are averaged across all primer pairs.

the number of *Taq*I sites) + (2 × the number of *Sal*I sites). If a variant was revealed by both *Taq*I and *Sal*I, it was conservatively assumed to be due to the same underlying mutation, and was counted as a single polymorphism.

For an individual locus, an average of 16 (of 40) enzymes recognized at least one cleavage site. Over all loci, 166 sites were observed in the approximately 8415 bp surveyed, and nine of these sites were polymorphic (5.4%). If each polymorphic restriction site is assumed to indicate a single nucleotide change within the recognition sequence, then 1.19% of the overall sequence surveyed was variable (range 0.0% to 3.09% across loci–Table 1).

Two of the loci, CM-01 and CM-28, revealed no RFLPs in surveys of 8.3% and 11.2% of their respective sequences. Among the five other loci assayed with comparable effort (Table 1), the nine restriction site polymorphisms revealed were distributed as follows: CM-39 and CM-45, one polymorphic restriction site each (*Hin*fI and *Alu*I, respectively); CM-12 and CM-14, two polymorphic sites each (*Alu*I, *Hin*dII and *Hin*fI, *Rsa*I, respectively) (Figure 2); and CM-67, three polymorphic sites (*Alu*I, *Cfo*I and *Dde*I).

Rare size deletions and insertions: To ascertain if moderate and low frequency polymorphisms may have gone undetected in the preliminary screening, an additional 36 chromosomes (18 individuals) for CM-14 and 18 chromosomes (nine individuals) for CM-45 were screened with enzymes that cleaved one or more sites in the initial survey. No further restriction site polymorphisms were found. However, a small (*ca.* 20-bp deletion) size variant was noted in the CM-45 region, as deduced from concordant fragment length changes in restriction profiles across several enzymes.

In the broader survey of all populations (see below),

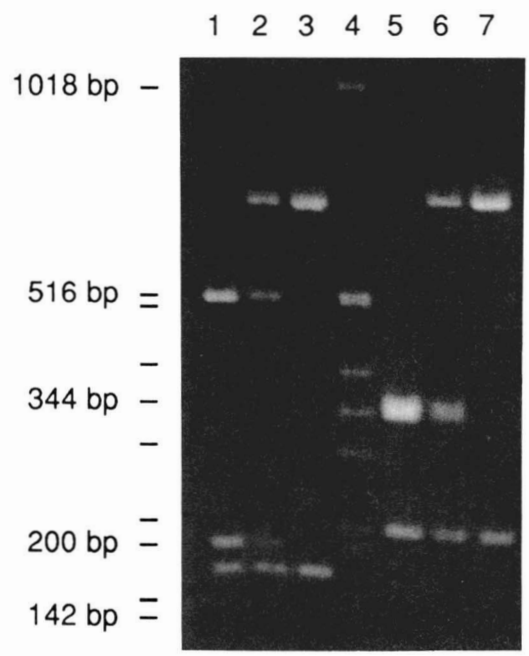

FIGURE 2.—Examples of restriction endonuclease profiles for locus CM-14. Lanes 1, 2 and 3 are individuals amplified with CM-14 and digested with *Hin*fI. Lane 4 is a size standard with selected fragment lengths indicated. Lanes 5, 6 and 7 are similar amplification products cut with *Rsa*I. The first lane in each set of three is homozygous for the presence of the polymorphic site, the second lane is heterozygous, and the third lane is homozygous for the absence of the polymorphic site.

two of the primer regions exhibited rare size polymorphisms, as follows: a 20-bp deletion in CM-45 was exhibited in heterozygous condition by two turtles from MEX-B ($N = 7$) and three from Galapagos ($N = 8$); a putative size variant in CM-45 (in two heterozygous individuals from Oman) may have been due to a small (10 bp) insertion; and a 20-bp insertion was found in CM-39 in a single heterozygous individual from Japan ($N = 19$). CM-39 also exhibited a large

TABLE 2

Observed phases of haplotypes for the loci exhibiting multiple restriction site polymorphisms

Primer	Haplotype[a] − −	− +	+ −	+ +	Unknown
CM-12	0	422	4	0	84
CM-14	0	181	87	209	36
CM-67A	234	0	0	102	138
CM-67B	262	53	127	0	32

[a] Haplotype designations for CM-12 refer to *Alu*I and *Hin*dII; for CM-14 *Hin*fI and *Rsa*I; for CM67A, *Alu*I and *Dde*I; and for CM-67B, *Alu*I and *Cfo*I.

(*ca.* 600 bp) deletion initially observed in the undigested amplified DNA from one MEX-A ($N = 7$), four Ascension ($N = 47$), and four Surinam ($N = 15$) individuals. All individuals appeared to be homozygous. Since it is highly unlikely that a rare polymorphism is present primarily in homozygous state, we suspect that only one of the chromosomes was amplified. We were unable to verify this possibility because the deleted region also contained the polymorphic restriction site. In any event, because all observed deletions and insertions were present in very low frequency, they were excluded from subsequent population analyses.

Phase determination for haplotypes with multiple site variants: For loci containing a single polymorphic restriction site (CM-39 and CM-45), haplotype designations are evident directly from the diploid genotype. This is not the case for loci with two or more polymorphic sites, since the *cis vs. trans* phase in double (or multiple) heterozygotes is not immediately apparent. Furthermore, the phase of double heterozygotes cannot be determined from gel profiles alone if the variant sites are separated by one or more invariant sites (QUINN and WHITE 1987).

For loci with multiple polymorphisms, we were able to circumvent this problem of phase determination by additional site linkage considerations. From the diploid genotypes of all individuals except double heterozygotes, each haplotype is evident from the genotype (*e.g.*, a "+ +/+ −" diploid consists of one "+ +" and one "+ −" haplotype). Table 2 shows the total numbers of such haplotypes for which phase was unambiguous. Consider, for example, CM-12. Only two of the four possible haplotype classes were present among the 426 unambiguous haplotypes (*i.e.*, no "− −" or "+ +" haplotypes were observed). This suggests that the presence *vs.* absence states of the *Alu*I and *Hin*dII site polymorphisms are in strong or complete disequilibrium, no doubt due to limitations on recombination because of tight physical linkage (the total length of the amplified region is only 1195 bp). Therefore, it is highly likely that the double heterozygotes are due to a combination of the "− +" and "+ −" phases. This assumption was then adopted to divide evenly the double-heterozygotes into the observed haplotype classes for subsequent analyses.

For CM-14, a similar rational was followed except that three of the four possible haplotypes were observed among the unambiguous diploid genotypes (Table 2). This raises the possibility that the unobserved haplotype, "− −," was present but only in double heterozygotes. There are two reasons why we believe that this is not the case, and that all double heterozygotes actually carried "− +" and "+ −" haplotypes. First, the observed number of double heterozygotes is not significantly different from the expectation based on the combined frequencies of "− +" and "+ −" haplotypes (including the unknown class; $\chi^2 = 2.46$, d.f. = 1). Second, by restriction site mapping we determined that the polymorphic sites in this region are adjacent, and therefore would produce different double-digestion fragment profiles for the *cis* and *trans* configurations (see arguments in QUINN and WHITE 1987). All double heterozygotes were examined in this manner and produced identical digestion patterns. Therefore we divided the unknown haplotypes evenly between the more common "− +" and "+ −" types.

The CM-67 primer region contained three polymorphic sites, and assignment of haplotypes was somewhat more complex. All pairwise genotype combinations were analyzed for disequilibria as before. CM-67 *Alu*I and *Dde*I sites showed a linkage relationship similar to that for CM-12, with only two of the four possible haplotypes observed among the unambiguous diploid classes (Table 2). As with CM-12, this indicates a strong disequilibrium among sites, such that haplotypes of double heterozygotes were divided evenly between the two observed phase classes. Next the *Alu*I (or *Dde*I, since they are in complete disequilibrium) and *Cfo*I sites were considered. These sites show a linkage pattern similar to that described above for CM-14 (Table 2), with three of the four possible haplotypes observed directly. In this case, if the double heterozygotes were a mixture of the "− +/+ −" and "+ +/− −" genotypes, we would expect most to be "− +/+ −" based on the allele frequencies. However, we were unable to determine whether all heterozygotes were identical, due to the large number of small fragments produced in double digestions. Since the "+ +" haplotype was never observed unambiguously, a conservative approach is to assume that all double heterozygotes were composed of the commonly observed haplotypes, and we partitioned them accordingly.

Population genetic analyses: Table 3 lists the haplotype frequencies and associated data. Considering only the polymorphic loci, mean heterozygosity per population ranged from 0.17 (MEX-B) to 0.44 (AUS),

TABLE 3

Allele frequency, mean sample size (*N*), direct count heterozygosity (h), average heterozygosity over all loci (H), average number of alleles (A), and percent loci polymorphic (P)

	Population[a]														
	Atlantic									Pacific					
Locus[b]	ASC	CSR	SUR	FLA	MEX-A	VEN	BRA	AFR	CYP	JAP	HAW	AUS	MEX-B	GAL	OMA
N	45.2	21.8	14.2	23.0	6.8	8.8	16.8	17.6	9.0	19.0	21.8	15.8	7.0	7.6	14.4
(SD)	(1.1)	(0.4)	(0.8)	(0.0)	(0.2)	(0.2)	(0.2)	(3.1)	(0.0)	(0.0)	(0.2)	(0.2)	(0.0)	(0.4)	(0.4)
CM-12															
− +	0.75	0.89	0.90	0.96	0.93	1.00	0.79	0.90	1.00	1.00	1.00	1.00	1.00	1.00	1.00
+ −	0.25	0.11	0.10	0.04	0.07	0.00	0.21	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
h	0.43	0.23	0.20	0.09	0.14	0.00	0.41	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CM-14															
− +	0.16	0.44	0.17	0.52	0.86	0.44	0.30	0.05	0.00	0.71	0.84	0.53	0.21	0.44	0.40
+ −	0.50	0.12	0.26	0.09	0.00	0.00	0.41	0.47	0.06	0.00	0.00	0.00	0.22	0.06	0.07
+ +	0.34	0.44	0.57	0.39	0.14	0.56	0.29	0.48	0.94	0.29	0.16	0.47	0.57	0.50	0.53
h	0.57	0.70	0.53	0.61	0.29	0.67	0.59	0.63	0.11	0.37	0.32	0.56	0.43	0.38	0.40
CM-39															
− −	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.06	0.00
− +	0.96	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.87	1.00	0.94	1.00
h	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.00	0.13	0.00
CM-45															
− −	0.77	0.77	0.73	0.70	0.79	0.56	0.65	0.66	0.67	0.79	0.59	0.50	0.14	0.25	0.40
− +	0.23	0.23	0.27	0.30	0.21	0.44	0.35	0.34	0.33	0.21	0.41	0.50	0.86	0.75	0.60
h	0.30	0.46	0.40	0.44	0.14	0.67	0.47	0.37	0.44	0.32	0.73	0.75	0.29	0.25	0.40
CM-67															
− −	0.67	0.19	0.47	0.33	0.29	0.50	0.94	0.58	0.28	0.69	0.88	0.28	0.93	0.75	0.43
− +	0.21	0.05	0.03	0.11	0.07	0.00	0.03	0.21	0.05	0.26	0.02	0.44	0.07	0.00	0.32
+ −	0.12	0.76	0.50	0.56	0.64	0.50	0.03	0.21	0.67	0.05	0.10	0.28	0.00	0.25	0.25
h	0.35	0.48	0.60	0.61	0.71	0.50	0.13	0.67	0.44	0.47	0.14	0.69	0.14	0.17	0.29
H	0.34	0.37	0.35	0.35	0.26	0.37	0.32	0.38	0.20	0.23	0.24	0.44	0.17	0.18	0.22
SD	0.08	0.12	0.11	0.13	0.12	0.15	0.11	0.13	0.10	0.10	0.14	0.14	0.08	0.06	0.09
A	2.4	2.2	2.2	2.2	2.0	1.6	2.2	2.2	1.8	1.8	1.8	2.0	1.8	2.0	2.0
SD	0.2	0.4	0.4	0.4	0.3	0.2	0.4	0.4	0.4	0.4	0.4	0.3	0.4	0.3	0.4
P	100	80	80	80	80	60	80	80	60	60	60	80	60	80	60

Data exclude nonvariable enzymes and loci.
[a] See legend to Figure 1 for location abbreviations.
[b] See footnote to Table 2 for allele designations.

with an overall average of 0.30 ± 0.09 (SE). In most cases, observed genotypic proportions within populations were in agreement with Hardy-Weinberg expectations based on observed haplotype frequencies: only three comparisons (among 75) showed significant deviations (at $P < 0.05$). These involved CM-67 in Oman (slight heterozygote deficit), and CM-45 in Hawaii and Australia (heterozygote excesses).

Table 4 presents estimates of the standardized variances in haplotype frequency across rookeries (F_{ST} values, corrected for sampling error) for each locus. On a global scale, interpopulation variation in allele frequencies is moderate to large (mean $F_{ST} = 0.17$), with χ^2 tests indicating significant population structure ($P < 0.05$) at all loci. Among rookeries within ocean basins, population structure was also significant overall, with mean F_{ST} values of 0.13 in both the Atlantic-Mediterranean and Indian-Pacific Oceans. Estimates of gene flow from F_{ST} yield mean values of $Nm = 1.7$ for green turtle rookeries within each of these ocean basins (Table 4).

Conventionally, Nm values much greater than one are interpreted as evidence of sufficient gene flow between populations to counter the effects of allele frequency divergence by genetic drift (but see ALLENDORF 1983), whereas values much less than one indicate severe restrictions on gene flow such that differentiation by genetic drift may be pronounced. Our estimated values of Nm (Table 4) reflect intermediate population connectedness. Such "gene flow" does not necessarily imply contemporary intercolony gene movement, but may in part reflect historical patterns of population interconnection (see beyond).

Nuclear haplotype frequencies were compared between particular pairs of populations that are of special interest because of geographic proximity, shared

TABLE 4

F_{ST}, χ^2, and associated *Nm* values for the surveyed nuclear loci in green turtles

	Atlantic-Mediterranean			Indian-Pacific			Global		
Locus	F_{ST}	χ^2	*Nm*	F_{ST}	χ^2	*Nm*	F_{ST}	χ^2	*Nm*
CM-12	0.049	16.46	4.9				0.089	45.39	2.6
CM-14	0.206	123.60	1.0	0.089	30.97	2.6	0.184	188.42	1.1
CM-39	0.020	5.56^{NS}	12.3	0.041	6.72^{NS}	5.8	0.050	24.10	4.8
CM-45	0.000		∞	0.154	26.80	1.4	0.120	61.20	1.8
CM-67	0.168	93.41	1.2	0.151	50.13	1.4	0.207	183.82	1.0
Overall	0.130	238.76	1.7	0.126	114.62	1.7	0.166	502.93	1.3

All test were significant at $P \leq 0.05$ unless otherwise indicated; NS = not significant.

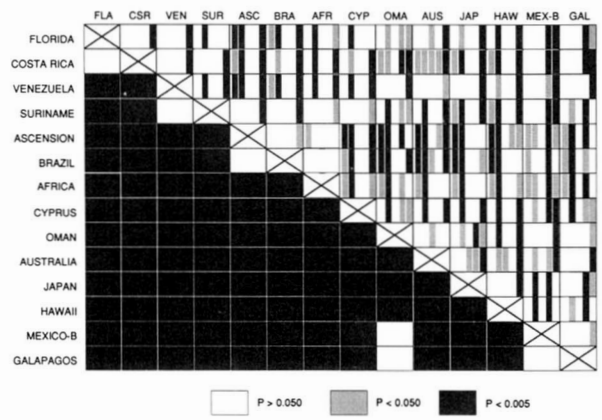

FIGURE 3.—Pairwise *G*-tests for significance of haplotype frequency differences between green turtle rookeries with regard to nDNA markers (above diagonal) and mtDNA markers (below diagonal). For nDNA, five columns representing the outcomes of statistical tests for each of the five assayed loci are shown for each inter-rookery comparison (for mtDNA, only a single such test is possible). The Quintana Roo (MEX-A) sample is not included because mtDNA data were unavailable.

feeding grounds, or prior documentation of a large mtDNA haplotype frequency difference. One dramatic case involves the Ascension Island and Surinam colonies. Turtles from these rookeries share feeding grounds along the northeastern coast of South America. Yet samples from the Ascension and Surinam populations exhibited fixed differences in mtDNA haplotype frequency, indicating strong natal site philopatry by females (BOWEN *et al.* 1992). However, at only one of the five nuclear loci (CM-67, Figure 3) did these rookeries differ significantly in haplotype frequency in the present study, and even at this locus all alleles were present in both populations (Table 3). For Ascension-Suriname, the mean estimate of *Nm* across assayed nuclear markers is $\cong 5$, indicating a moderate level of nuclear gene flow or recent historical connectedness. Notably, the Ascension and Surinam rookeries share a rare 600 bp deletion in CM-39 that is virtually absent in other Atlantic study sites. A similar example involves the Florida and Surinam rookeries, which share no mtDNA haplotypes but are quite similar in frequencies of nuclear haplotypes (Table 3; Figure 3), with an estimated mean $Nm \cong 7$.

A few rookery populations were essentially indistinguishable in mtDNA assays (BOWEN *et al.* 1992), and these proved to show nonsignificant differences with regard to most nuclear markers as well. For example, the Florida and Costa Rica samples were nearly monomorphic for the same mtDNA haplotype, and only one of the five nuclear markers showed statistically significant differences ($P < 0.05$). Similarly, two other pairs of populations (Brazil/Ascension and MEX-B/Galapagos) exhibited high frequencies of the same respective mtDNA haplotypes in addition to indicating nonsignificant differences at four of five of the nuclear loci (Figure 3).

Although these multiple tests of the pairwise significance of haplotype frequencies between rookery populations are not statistically independent, it is noteworthy that only 28% of the 455 nuclear RFLP comparisons yielded significant differences at the $P < 0.005$ level. In contrast, 93% of the 91 mtDNA comparisons were significantly different at this same probability level (Figure 3). Similar qualitative conclusions can be drawn about inter-rookery comparisons within either the Atlantic-Mediterranean or Indian-Pacific Ocean basins (Figure 3). In general, many population samples exhibited fixed or nearly fixed differences in mtDNA haplotypes, but the same nuclear haplotypes typically were shared by nearly all populations (Table 3). Thus pairwise F_{ST} values for nuclear markers typically yield estimates of *Nm* greater than one, whereas those for mtDNA were usually near zero (BOWEN *et al.* 1992).

DISCUSSION

The PCR amplification approach to RFLP identification: This new PCR approach to RFLP analysis has revealed 12 nuclear DNA polymorphisms in a global survey of green turtle populations. Once suitable polymorphisms were identified, we found this method to provide a rapid and convenient procedure for the survey of Mendelian polymorphisms in large numbers of individuals. However, considerable ex-

pense (both in time and money) was involved in the initial effort to identify the polymorphisms. Based on our experience with this PCR-based approach in the green turtle analysis, we can offer the following comments regarding its strengths and weaknesses.

On the negative side, several months were required for the cloning and screening of single-copy DNA sequences. A total of 15 primer pairs was sequenced and synthesized (at considerable expense), of which seven successfully amplified only the targeted region. Two of these seven working primer pairs proved monomorphic based on the 40 restriction endonucleases employed, although more sensitive screening methods such as denaturing gradient gel electrophoresis or DNA sequencing would quite likely reveal additional polymorphisms at these loci.

On the positive side, this technique has several advantages over the more standard Southern blotting approach to RFLP analysis. First, only a small amount of nDNA is required for the PCR reaction, and assays can be conducted even when tissue samples are minute (*e.g.*, muscle scrapes from a turtle plastron), partially degraded (as was the case for the MEX-A samples in this study) or in limited availability (*e.g.*, blood from an endangered species). Second, since the DNA analyzed is bounded by primers and therefore is of a defined length, restriction site variants can be distinguished unambiguously from insertion/deletion variants due to the required conservation of the total fragment size. Third, since the DNA is amplified *in vitro*, restriction fragment changes cannot be due to variation in methylation patterns of the genomic DNA (amplified DNA is completely unmethylated). Finally, since large amounts of DNA can be produced by amplification, the PCR-based method eliminates the need for radionucleotides and the long exposure times (up to seven days) often required to produce a signal with autoradiography. PCR-amplified DNA can be digested directly and visualized in EtBr-stained agarose gels in just a few hours.

Another advantage of either the PCR-based or Southern-based approaches involves the potential availability of RFLPs from a nearly limitless pool of nuclear gene regions (including noncoding sequences as well as both silent and replacement variation in coding sequences). This contrasts with traditional allozyme methods, where normally less than 100 structural genes can be assayed (for replacement substitutions only) with available histochemical stains. Furthermore, additional information about the genetic composition of particular haplotypes can stem from nuclear RFLP approaches, especially when determination of the gametic phase of multiple polymorphisms is feasible (as was true here for loci CM-12, CM-14 and CM-67–Table 2). Against these appealing aspects of RFLP approaches stands the counterpoint that allozyme assays are faster, less expensive, require much less molecular expertise, and can furnish qualitatively similar kinds of genetic information, provided sufficient polymorphisms are found.

Nuclear DNA polymorphisms and the natural history of green turtles: Among the dozen RFLPs revealed in our assays, nine (involving five independent gene regions) represented common restriction site polymorphisms. These all exhibited simple Mendelian behavior, and form the basis for the population genetic conclusions that follow.

First, genotypic proportions within nesting beach populations conformed to Hardy-Weinberg expectations in 72 of 75 cases. Thus if significant inbreeding or other genetic substructure occurs within such populations, it was beyond resolution of this study. Second, we observed significant differentiation in haplotype frequencies globally, as well as within both the Atlantic-Mediterranean and Indian-Pacific Ocean basins. Third, common haplotypes at all assayed nuclear loci were shared by virtually all rookeries around the world, and many particular pairs of rookeries were not significantly different in haplotype frequency. Fourth, this pattern of global sharing of nuclear haplotypes among rookeries contrasts sharply with prior results from mtDNA analyses, where samples from many rookeries proved distinguishable from most others by fixed or nearly fixed haplotype frequency differences, and a relatively deep phylogenetic split cleanly distinguished all Atlantic-Mediterranean rookeries from those in the Indian-Pacific Ocean basins (BOWEN *et al.* 1992).

What could account for the apparent differences in the population genetic structure in nuclear *vs.* mtDNA assays? One genetic consideration (here as well as with allozyme studies) is a difference in rate of evolution for the mitochondrial *vs.* the nuclear genomes. In comparison to typical single copy nuclear sequences, mtDNA sequences are thought to evolve rapidly in vertebrates (BROWN, GEORGE and WILSON 1979) [though perhaps at a pace somewhat slower in marine and other turtles (AVISE *et al.* 1992)]. The geographic distribution of genetic polymorphisms indicates that most mtDNA differences observed between green turtle rookeries likely are attributable to *de novo* mutations, whereas the observed nDNA differences primarily reflect shifts in frequencies of preexisting (ancestral) alleles. This is suggested by the fact that for mtDNA the between-ocean genetic divergence is much greater than that within oceans, and many haplotypes are highly localized, whereas most nDNA haplotypes occurred at nearly all rookeries.

A second genetic consideration is the expected difference in effective population size for nuclear *vs.* mtDNA haplotypes. Because mtDNA is effectively haploid in transmission and predominantly maternally

inherited, it is expected to exhibit a fourfold lower effective population size than is an autosomal nuclear gene (all else being equal). Thus mtDNA frequencies should be more strongly influenced by the effects of genetic drift and population bottlenecks, and could exhibit a larger interlocality variance for these reasons. This same pattern could also result from sampling effects alone. However, our sample sizes for the (diploid) nDNA haplotypes were in general twice as large as those for the (haploid) mtDNA, so the statistical power to detect significant differences in nDNA between rookeries actually was enhanced. Thus the differences in our sample sizes between nuclear and mitochondrial haplotypes cannot account for the stronger tendency of mtDNA to exhibit significant interpopulational differences (Figure 3). Overall however, the differences in inheritance dynamics between nDNA and mtDNA, as well as the presumed differences in mutation rate, are both consistent with qualitative distinctions between the mtDNA and nDNA data sets.

A final influence contributing to the differences in population structure revealed in the nDNA *vs.* mtDNA assays may involve life history differences between male and female green turtles. Based on the nearly fixed mtDNA haplotype differences which characterize most rookeries (BOWEN *et al.* 1992), a strong tendency for natal site philopatry by females is indicated. Based on the comparatively low level of interpopulational differentiation characterizing most surveyed nDNA loci, interpopulation gene flow (or historical connectedness) mediated by males is suspected. Since our estimates of nDNA haplotype frequencies are based on egg or hatchling samples, results cannot be attributed to inclusion of males from other rookeries. However, results might reflect interpopulational gene flow mediated by matings in areas where turtles from two or more rookeries co-occur.

There is little other information available on green turtle mating patterns to support this hypothesis, beyond the observation that matings may occur on feeding grounds or during migration, as well as adjacent to nesting beaches. Female green turtles may be able to store and utilize sperm for long periods of time (perhaps months or years), as is known for several other turtle species (OLIVER 1955). Thus during the extensive migrational movements of adult green turtles, it is quite plausible that matings between individuals from different rookeries, coupled with female natal site philopatry, could provide an avenue of interrookery exchange for nuclear genes that is essentially closed to mtDNA. Even if males should also prove to be natal site philopatric in migratory behavior, matings taking place at other times and locations could furnish a ready opportunity for interpopulational exchange of nuclear genes, at least among rookery populations that overlap in feeding territories or migrational corridors.

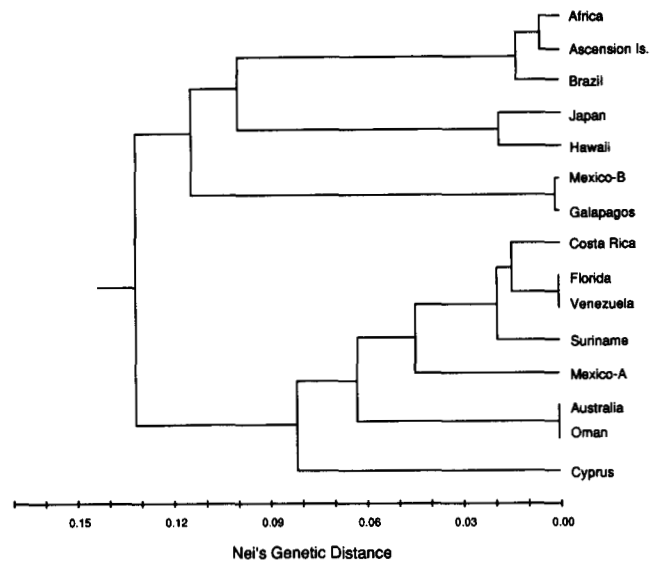

FIGURE 4.—UPGMA dendrogram for the 15 green turtle rookeries. The cophenetic correlation is 0.58.

Biogeographic relationships among rookeries: Can the observed nDNA haplotype frequencies be interpreted in terms of the suspected relationships of the rookeries based on zoogeographic evidence? Figure 4 presents a UPGMA phenogram based on nDNA genetic distances derived from haplotype frequencies at the five polymorphic marker loci. Some of the broader genetic clusters suggest implausible evolutionary relationships. These involve the grouping of Ascension, Africa and Brazil with Japan and Hawaii, and the grouping of Australia and Oman with other Atlantic populations. Since all observed changes in genetic distance were attributable to shifts in frequencies of alleles, such counterintuitive clusters are nearly inevitable when data from only a few polymorphic loci and many rookeries are considered.

On the other hand, several of the genetic clusters appear highly plausible, including the close associations indicated for: (a) Ascension Island, Africa and Brazil, which are closely related based on mtDNA evidence, and are proximate geographically (BOWEN *et al.* 1992); (b) Florida, Venezuela, Costa Rica, Suriname and Mexico-A, all of which are in or adjacent to the Caribbean Sea; (c) Mexico-B and Galapagos, which are geographically close and considered by some to be a distinct taxonomic form (the black turtle, *C. m. agassizi* or *C. agassizi*); (d) Japan and Hawaii, in the central and western regions of the north Pacific; and, perhaps, (e) Oman and Australia. Most of these groupings also appeared consistently in cluster analyses based on the other genetic distance measures in BIOSYS-1 (analyses not shown). Thus in general, there remains a reasonably strong association between the geographic proximity of green turtle rookeries

and their genetic relationship at assayed nuclear RFLP loci (Figure 4). This genetic association between nearby rookeries must be due either to recent historical connectedness, or to contemporary gene flow via inter-rookery matings. If the latter explanation is correct, males provide an avenue for inter-rookery exchange of nDNA that is largely closed to mtDNA (due to the propensity for natal-homing by females).

In summary, population genetic structure in green turtles has distinctive biparental (nDNA) and maternal (mtDNA) components, probably arising at least in part from the mating system coupled with characteristic natural histories for males *vs.* females. Apparently, different population genetic structures can co-exist within a single organismal lineage.

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APPENDIX

Table 5 gives the sequences of the anonymous primers (5′ to 3′) used in this study. Names refer to the genomic clones from which they were derived and "L" and "R" designations refer to arbitrarily assigned left and right ends.

TABLE 5

Sequences of the anonymous primers (5′ to 3′) used in this study

Primer	Sequence	Copy class
pdCM-1R	5′-GGAAGCTTTCAAGCTACACTGT-3′	Single
pdCM-1L	5′-GGAAGCTTCATAACACAAAACC-3′	
pdCM-12R	5′-AGCTGAAGCCAATGAAGAAGAA-3′	Single
pdCM-12L	5′-GCTCAGGTTTAGCTCGAAGGT-3′	
pdCM-14R	5′-TAAGCATTATACGTCACGGA-3′	Low
pdCM-14L	5′-AGTATTTGGGCAGAACAGAA-3′	
pdCM-28R	5′-TAAATGCCAGGTATGTAACTC-3′	Single
pdCM-28L	5′-GATTGCTGGTCTCTGGAAGGCT-3′	
pdCM-39R	5′-TGCTAGTTTTGTTAGTTCTGGT-3′	Single
pdCM-39L	5′-ATAGTGGATTGGAGAAGTTGTT-3′	
pdCM-45R	5′-CTGAAAGTGTTGTTGAATCCAT-3′	Single
pdCM-45L	5′-CCGCAAGCAAAACATTCTCT-3′	
pdCM-67R	5′-GAATATAAGATTTCATACCCCA-3′	Low
pdCM-67L	5′-TTTAATTCTGAAAACTGCTCTT-3′	