Geographic Structure of Mitochondrial and Nuclear Gene Polymorphisms in Australian Green Turtle Populations and Male-Biased Gene Flow

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ABSTRACT

The genetic structure of green turtle (*Chelonia mydas*) rookeries located around the Australian coast was assessed by (1) comparing the structure found within and among geographic regions, (2) comparing microsatellite loci *vs.* restriction fragment length polymorphism analyses of anonymous single copy nuclear DNA (ascnDNA) loci, and (3) comparing the structure found at nuclear DNA markers to that of previously analyzed mitochondrial (mtDNA) control region sequences. Significant genetic structure was observed over all regions at both sets of nuclear markers, though the microsatellite data provided greater resolution in identifying significant genetic differences in pairwise tests between regions. Inferences about population structure and migration rates from the microsatellite data varied depending on whether statistics were based on the stepwise mutation or infinite allele model, with the latter being more congruent with geography. Estimated rates of gene flow were generally higher than expected for nuclear DNA (nDNA) in comparison to mtDNA, and this difference was most pronounced in comparisons between the northern and southern Great Barrier Reef (GBR). The genetic data combined with results from physical tagging studies indicate that the lack of nuclear gene divergence through the GBR is likely due to the migration of sGBR turtles through the courtship area of the nGBR population, rather than male-biased dispersal. This example highlights the value of combining comparative studies of molecular variation with ecological data to infer population processes.

DIFFERENCES between sexes in the rate of gene flow between populations are expected to result in different distributions of genetic variation among populations for maternally *vs.* biparentally inherited molecular markers. With male-biased gene flow, the proportion of genetic variance among populations should be greater for maternally inherited mitochondrial DNA (mtDNA) than for biparentally inherited nuclear markers, after adjustment for differences in effective population size (BIRKY *et al.* 1989; *e.g.*, PALUMBI and BAKER 1994). However, comparisons between mtDNA and nuclear genes are confounded if there are consistent differences among different classes of nuclear gene markers or if mutational processes conflict with the assumptions inherent in estimating gene flow. For example, several studies have reported different levels of population structure between allozyme and anonymous single-copy nuclear (ascnDNA) loci (KARL and AVISE 1992; ZHANG *et al.* 1993; POGSON *et al.* 1995), among classes of hypervariable loci (SCRIBNER *et al.* 1994; ALLEN *et al.* 1995; DALLAS *et al.* 1995), and among different sets of ascnDNA loci (KARL and AVISE 1992; MCDONALD *et al.* 1996). Given that all nuclear loci should respond similarly to a drift-migration equilibrium, such differences may reflect the effects of selection (*e.g.*, KARL and AVISE 1992) or variation in mutation rates or processes (SLATKIN 1995a).

A prominent example of the detection of male-mediated gene flow through comparison of mitochondrial and nuclear polymorphism involved green turtles, *Chelonia mydas* (KARL *et al.* 1992). It has long been recognized from recaptures of tagged adults that females frequently return to the same or nearby nesting sites within and between seasons (CARR *et al.* 1978; LIMPUS *et al.* 1984; LIMPUS *et al.* 1992). This raised the possibility that females were returning to their natal population after extensive migrations over some 30–50 years of development (LIMPUS and WALTER 1980). Natal homing to distinct geographical regions was confirmed by the demonstration of heterogeneity of mtDNA allele frequencies among populations on both global (BOWEN *et al.* 1992) and regional (NORMAN *et al.* 1994; ENCALADA *et al.* 1996) scales. In contrast, movements by males are less well studied (but see DIZON and BALAZS 1982; GREEN 1984; LIMPUS 1993) and available ecological evidence does not address natal site fidelity of breeding males. To address this question, KARL *et al.* (1992) examined the structure of polymorphisms at biparentally inherited ascnDNA loci in hatchlings from different rookeries on a global scale. This revealed much lower levels of heterogeneity among populations than observed in mtDNA, with only 28% of pairwise tests reveal-

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ing heterogeneity of ascnDNA allele frequencies compared to 93% for mtDNA. Although it was noted that expected differences in effective population size and mutation rate could contribute to greater geographic structuring of mtDNA variation, the results were interpreted as evidence for male-biased gene flow arising from differences in the natural history of males and females. However, a direct test, which examined population-specific mtDNA markers in breeding males, demonstrated that male green turtles, like females, are philopatric (FITZSIMMONS *et al.* 1997).

To investigate this situation further, we have developed microsatellite markers for green turtles (FITZSIMMONS *et al.* 1995) and applied them to populations previously studied for mtDNA variation (NORMAN *et al.* 1994) and that are the subject of a long-term tagging program (LIMPUS *et al.* 1992; PRINCE 1994). The properties of microsatellite loci are becoming well known and, increasingly, they are being used to assess population genetic structure at nuclear loci (reviewed in DOWLING *et al.* 1996; JARNE and LAGODA 1996). A major advantage of microsatellites over restriction fragment length (RFLP) studies of ascnDNA loci is that the mutation rate for microsatellites is relatively high (10^{-2}–10^{-4}, WEBER and WONG 1993), which increases the ability to track coalescence events and detect population-specific alleles. Because most mutations are expected to generate previously existing allele sizes and to be dominated by additions or deletions of one copy of the tandem repeat, it has been recognized that the infinite allele mutation model (IAM) that underlies most population genetic models is probably inappropriate (SLATKIN 1995a; JARNE and LAGODA 1996). This has led to development of new measures of population subdivision and equilibrium migration rates (*e.g.*, R_{st}, SLATKIN 1995a) based on the stepwise mutation model (SMM) originally developed for allozymes (OHTA and KIMURA 1973), but allowing for occasional mutations of larger effect (DI RIENZO *et al.* 1994). R_{st} is an analog of NEI's (1973) G_{st} and is derived from variances in allele size. Estimates of R_{st} are expected to be larger than the corresponding IAM statistic, F_{st}, and to reflect migration rates more accurately if mutation rates are high and coalescence times are substantially smaller within than among populations (*i.e.*, τ/N is large or Nm is small; SLATKIN 1995a). However, the validity of this approach has been questioned where there are constraints on allele size (NAUTA and WEISSING 1996) and there have been few empirical comparisons of alternative statistics for microsatellites (but see VALSECCHI *et al.* 1997). Nor have there been comparisons made between the distribution of variation at microsatellite *vs.* other ascnDNA loci.

Recaptures of tagged females from east Australian rookeries indicate that nesting females display high fidelity to regional nesting beaches but, within a region, some females nest on more than one beach (LIMPUS *et al.* 1984; LIMPUS *et al.* 1994). Additionally, tagging data (LIMPUS *et al.* 1992; PRINCE 1994) have shown extensive overlaps of different nesting populations at widely dispersed feeding grounds (see Figure 1). In agreement with the tagging data, NORMAN *et al.* (1994) uncovered significant differences in mtDNA allele frequencies between each of the four regional nesting populations; *i.e.*, the southern Great Barrier Reef (sGBR), northern Great Barrier Reef (nGBR), Gulf of Carpentaria (GoC), and Western Australia (WA) (see Figure 1), but no significant genetic structuring was detected among rookeries within these regions. Two mtDNA haplotypes (A and B) and closely related variants were restricted to Pacific Ocean rookeries, whereas a third divergent haplotype (C) and its variants were restricted to the Gulf and Indian Ocean rookeries. This phylogeographic division between the Pacific Ocean (*i.e.*, nGBR and sGBR) and Indian Ocean rookeries appears to reflect a long-term separation, with the GoC rookery being recently (<9500 years, JONES and TORGERSEN 1988) established by colonization from the west (NORMAN *et al.* 1994; C. MORITZ, C. LIMPUS and R. SLADE, unpublished results).

Our study focuses on green turtle rookeries located around the Australian coast and extends the analysis of KARL *et al.* (1992) by (1) incorporating hierarchical sampling and comparing variation among rookeries within a region *vs.* among geographic regions, (2) comparing geographic structure detected at microsatellite loci *vs.* RFLP analyses of ascnDNA loci, and (3) focusing on a smaller geographic region including populations for which there is extensive information from ecological tagging studies. The combination of tag-return and mtDNA data indicates that the Australian green turtle rookeries provide an excellent opportunity to investigate the generality of differences in mtDNA *vs.* nuclear DNA (nDNA) variation and to investigate the mechanisms responsible. If the differences reported by KARL *et al.* (1992) are due primarily to a lower sensitivity of RFLP analysis of ascnDNA loci, then we should observe more population subdivision for microsatellites than for ascnDNA loci, although this may depend on the mutational model and statistic used (*i.e.*, IAM F_{st} *vs.* SMM R_{st}). If there is a substantial difference in patterning of mtDNA *vs.* nDNA loci, then we should be able to put this into the context of male-mediated gene flow, as suggested by KARL *et al.* (1992), through opportunities for mating of turtles during breeding migrations, overlap in feeding ground distributions, or relaxed philopatry of males. We hypothesize that the low number of alleles previously observed at ascnDNA loci may have prevented the detection of genetic heterogeneity at nuclear loci relative to that of mtDNA. Specifically, we test whether microsatellite markers outperform ascnDNA loci as indicators of population structure and gene flow and whether results from either set of nuclear markers can be interpreted relative to the available ecological data.

MATERIALS AND METHODS

Sampling: The sampling design allowed for comparisons of rookeries within regions (n = 9) located between 57 and

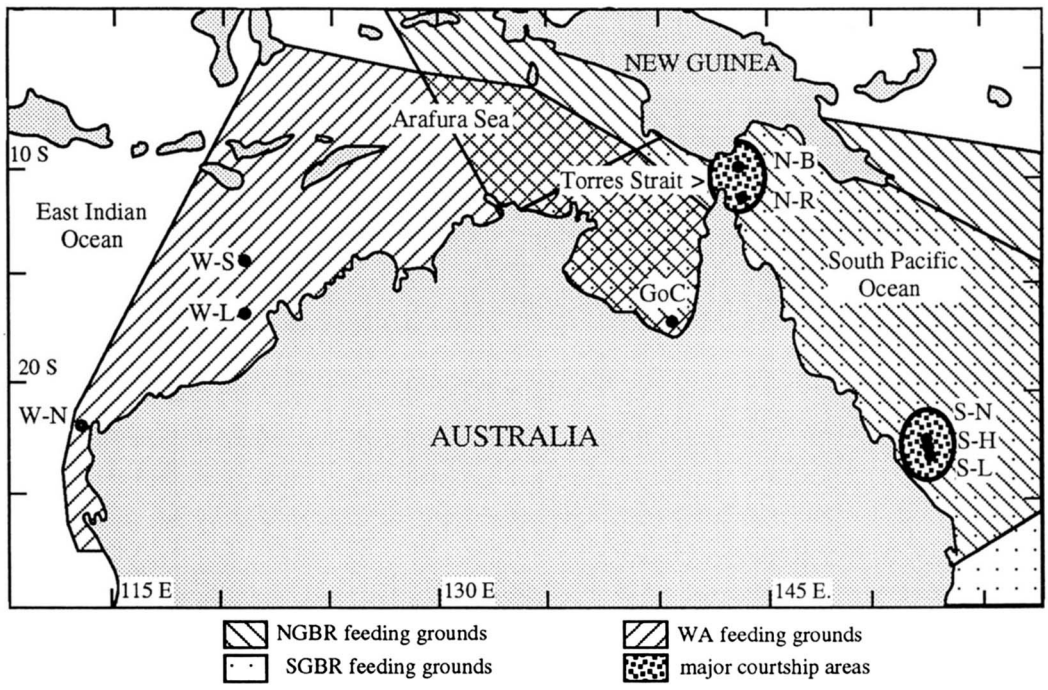

FIGURE 1.—Sample locations of green turtle (*Chelonia mydas*) populations in four regions of Australia, indicating courtship areas and overlapping feeding grounds. Some sGBR and nGBR females also share feeding grounds off New Caledonia and sGBR females have been captured in feeding grounds in Fiji. Abbreviations are as follows: for Western Australia: W-N, North West Cape; W-S, Sandy Island; and W-L, Lacepede Island; for Gulf of Carpentaria: GoC, Bountiful Island; for northern Great Barrier Reef: N-B, Bramble Cay; N-R, Raine Island; for southern Great Barrier Reef: S-N, North West Island; S-H, Heron Island; S-L, Lady Musgrave Island. The extent of feeding grounds used by GoC breeding turtles is unknown.

900 km apart, as well as comparisons between regions (n = 4) located 1000 to 4700 km apart (Figure 1). Sample locations of the nine rookeries were as follows: in WA, Lacepede Island, North West Cape, and Sandy Island; in the GoC, Bountiful Island; in the nGBR, Bramble Cay and Raine Island; and in the sGBR, North West Island, Heron Island, and Lady Musgrave Island. Liver or muscle samples taken from nonsibling hatchlings for a previous study of mtDNA variation (NORMAN *et al.* 1994) constituted the majority of the samples analyzed. Additional samples from Bountiful Island (GoC) were obtained by collecting blood (0.5–1.0 ml) from the dorsal cervical sinus of courting adult males and females. Blood was collected into a lysis buffer (100 mM Tris, 100 mM EDTA, 10 mM NaCl, 0.5% SDS) and genomic DNA was extracted (extraction buffer: 10 mM Tris, 1 mM EDTA, 10 mM NaCl, 0.1% SDS) by a salting-out procedure (see FITZSIMMONS *et al.* 1995). Total sample size per locus for microsatellites averaged 275 animals. The analysis of ascnDNA loci was restricted to between-region comparisons and sample sizes were ~20 individuals per region, or 80 overall. mtDNA control region sequence data from NORMAN *et al.* (1994) and C. MORITZ, C. LIMPUS and R. SLADE (unpublished results) were used to estimate levels of population subdivision and gene flow.

Microsatellite loci: Four dinucleotide (CA)n microsatellite loci previously found to be variable in green turtles (Cm3, Cm58, Cm72, and Cc117) were amplified from genomic DNA by PCR using either α^{32}P or α^{33}P dATP incorporation (FITZSIMMONS *et al.* 1995). Each locus contains an uninterrupted (CA)n repeat array and analysis of maternal alleles from clutches (950 offspring from 13 females) have revealed both single and multistep mutations, usually to preexisting allele sizes (N. FITZSIMMONS, unpublished results). Typically 0.05 μCi of radiation was added to 12.5 μl reaction volumes with final concentrations of 20 μM dATP, 200 μM each of the remaining dNTPs (G, T, C), 2–3 mM $MgCl_2$, 0.5 μM primer, 5–50 ng of template DNA, 1× Taq buffer and 0.25 units of Taq polymerase. PCR products were run through 6% denaturing sequencing polyacrylamide gels and visualized by autoradiography. Allele lengths were determined by comparison to sequenced size standards of known length that were run in the middle and near the edges of each gel. Any ambiguities in scoring were checked by rerunning either the original or a new PCR product.

Anonymous single copy nuclear loci: We analyzed variability among regions at five ascnDNA loci previously found to be variable in green turtles: Cm12, Cm14, Cm39, Cm45, and Cm67 (KARL *et al.* 1992; KARL and AVISE 1993). Loci were amplified by PCR methods following the procedures of KARL *et al.* (1992), but without the addition of bovine serum albumin. PCR products (5 μl) were checked for correct amplification by electrophoresis through 1.2% agarose gels. Products were digested with restriction enzymes previously found to reveal variation (KARL *et al.* 1992), electrophoresed through 2.5% agarose gels, and stained with ethidium bromide. Locus-specific digests were as follows: Cm12, *Alu*I and *Hin*dII; Cm14, *Hin*fI and *Rsa*I; Cm39, *Hin*fI; Cm45, *Alu*I and *Hin*fI; and Cm67, *Cfo*I and *Dde*I. Following KARL *et al.* (1992), loci were designated as the entire PCR product and alleles were scored as haplotypes. For example, an individual scored as "+−/+−" at Cm67 would be homozygous for presence of the restriction site at *Cfo*I and homozygous for absence of the restriction site at *Dde*I but would be scored as having two "+−" haplotypes in tests of population structure. The *cis vs. trans* phase of double heterozygotes was determined through site linkage analysis as done by KARL *et al.* (1992).

Statistical analyses: Observed heterozygosity and number of alleles per locus were calculated for each rookery and expected heterozygosity was estimated by

$$H_{\text{exp}} = 1 - \sum_{i=1}^{k} p_i^2 \qquad (1)$$

where p is the frequency of the ith allele for each of k alleles

(NEI 1987, p. 177). Linkage disequilibrium between loci and deviations from HARDY-WEINBERG equilibrium were tested by a Markov chain method (2000 dememorizations, 50 batches, 1000 iterations) for each rookery with the program GENEPOP (RAYMOND and ROUSSETT 1995). Divergence in allele frequencies among rookeries and among regions was tested by comparing observed chi-squared values with 10,000 randomized data sets generated by REAP (MCELROY *et al.* 1992). These tests of allele frequency divergence were performed for comparison with the previous results of KARL *et al.* (1992).

Population subdivision was analyzed under two models of mutation as appropriate, wherein F_{st}-based estimates assume an IAM and R_{st}-based estimates assume the SMM. We calculated WEIR and COCKERHAM's (1984) F_{st} estimator (θ) in the FSTAT program (GOUDET 1994) by Jackknifing procedures at each locus and permutation tests to determine significance. R_{st} (SLATKIN 1995a) was estimated from the microsatellite data as

$$R_{st} = \frac{\bar{S} - S_w}{\bar{S}} \quad (2)$$

where S_w is twice the average of the estimated variances in allele size within each population and $\bar{S}$ is twice the estimated variance in allele size in the collection of populations together (SLATKIN 1995a). We obtained R_{st} values using R_{st}CALC (GOODMAN 1997), which calculates unbiased estimates by standardizing allele size at each locus, taking into account unequal sample sizes, and tests significance by permutation methods ($n = 3000$). To partition molecular variance at different hierarchical levels, we estimated Φ statistics in AMOVA (EXCOFFIER *et al.* 1992) for the ascnDNA and mtDNA markers under a model of equal genetic distance in which Φ_{st} functions as an analogue of θ (WEIR and COCKERHAM 1984). For the microsatellites, we used the squared differences in allele lengths to form the distance matrix (MICHALAKIS and EXCOFFIER 1996) and derive Φ_{st} as an analogue of SLATKIN's (1995a) R_{st}.

Pairwise estimates of F_{st} and R_{st} between regions were made to address specific questions about gene flow between regions and to compare the values to ecological data. Gene flow (Nm) was estimated for the nuclear data from F_{st} by the equation

$$Nm = \frac{1}{4}\left(\frac{1}{F_{st}} - 1\right) \quad (3)$$

and for R_{st} by

$$Nm = \frac{d_s - 1}{4d_s}\left(\frac{1}{R_{st}} - 1\right) \quad (4)$$

where d_s is the number of populations compared (SLATKIN 1995a). For the mtDNA data, Nm_f was estimated by

$$Nm_f = \frac{1}{2}\left(\frac{1}{F_{st}} - 1\right) \quad (5)$$

For all genomic markers, significance levels for tests involving multiple comparisons were adjusted following the sequential Bonferroni procedure of RICE (1989) unless otherwise stated.

RESULTS

Microsatellite loci: The microsatellite loci proved to be highly variable (Figure 2). The most variable locus was Cm72, with 42 alleles overall, and the least variable was Cm58, with 12 alleles. Individual rookeries had an average of 10.8–19.3 alleles per locus and a significant relationship existed between the mean number of alleles and mean sample size ($r^2 = 0.57$, $P = 0.02$, d.f. = 8). Heterozygosity was high in each rookery ($H \geq 0.81$; Table 1) and no correlation was found between heterozygosity (h) and sample size across rookeries at any locus ($r^2 < 0.45$, $P > 0.05$, d.f. = 8). Among the four regions, the number of alleles per locus and levels of heterozygosity were quite similar, except for somewhat lower values in the less-sampled GoC population (Table 1). Linkage disequilibrium was not observed between any loci in any rookery, $P > 0.05$, without correction for multiple tests. Agreement with expectations of HARDY-WEINBERG equilibrium was found for all loci except Cm72 at Heron Island (sGBR) and Lacepede Island (WA), and Cm58 at Heron Island ($P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively, after adjusting for $n = 36$ tests). In each of those tests, fewer heterozygotes were observed than expected.

Unique alleles were observed in most rookeries (eight of nine), in each region, and within each ocean basin, and accounted for a low to moderate proportion of the total observed alleles (10.6, 15.4, and 22.1%, respectively). At each locus, an average of 0.026 ± 0.004 alleles were unique per rookery. There was a marked tendency for unique alleles to occur at the extremes of the size distribution (Figure 2) and there was no correlation ($r^2 = 0.01$, d.f. = 8, $P = 0.76$) between sample size and number of unique alleles at a rookery. None of the alleles unique to the Indian Ocean were shared by both the WA and GoC populations, whereas 40% of the alleles unique to the Pacific Ocean were found in both the nGBR and sGBR regions.

Allele frequency differences among rookeries within regions were not significant except at the Cm72 and Cc117 loci in the WA rookeries ($P = 0.023$ and 0.003, respectively). Pairwise comparisons of allele frequencies at the three rookeries indicated that Sandy Island was distinct from Lacepede Island at both loci (Cc117, $P = 0.0002$; Cm72, $P = 0.009$), whereas the Lacepede Island and North West Cape rookeries were statistically homogeneous (Cc117, $P = 0.30$; Cm72, $P = 0.71$). Given these results, data for Sandy Island were excluded from analyses of genetic differentiation between WA and the other regions. In contrast to the other rookeries, Sandy Island occurs off the continental shelf and may either be genetically isolated or influenced by gene flow from Indonesian rookeries to the north. In agreement with the previous mtDNA data (NORMAN *et al.* 1994), rookeries within regions showed no genetic subdivision at individual microsatellite loci, or across combined loci, and F_{st} and R_{st} values were not significantly different from zero ($P > 0.05$).

RFLP analysis of ascnDNA loci: RFLP digests of ascnDNA loci yielded a total of seven polymorphic restriction sites as follows: one polymorphic site each at Cm12 and Cm39 (*Hin*dII and *Hin*fI, respectively), two at Cm67 (*Cfo*I and *Dde*I), and three at Cm14 (*Rsa*I and two sites at *Hin*fI) (Table 2). No polymorphic sites were

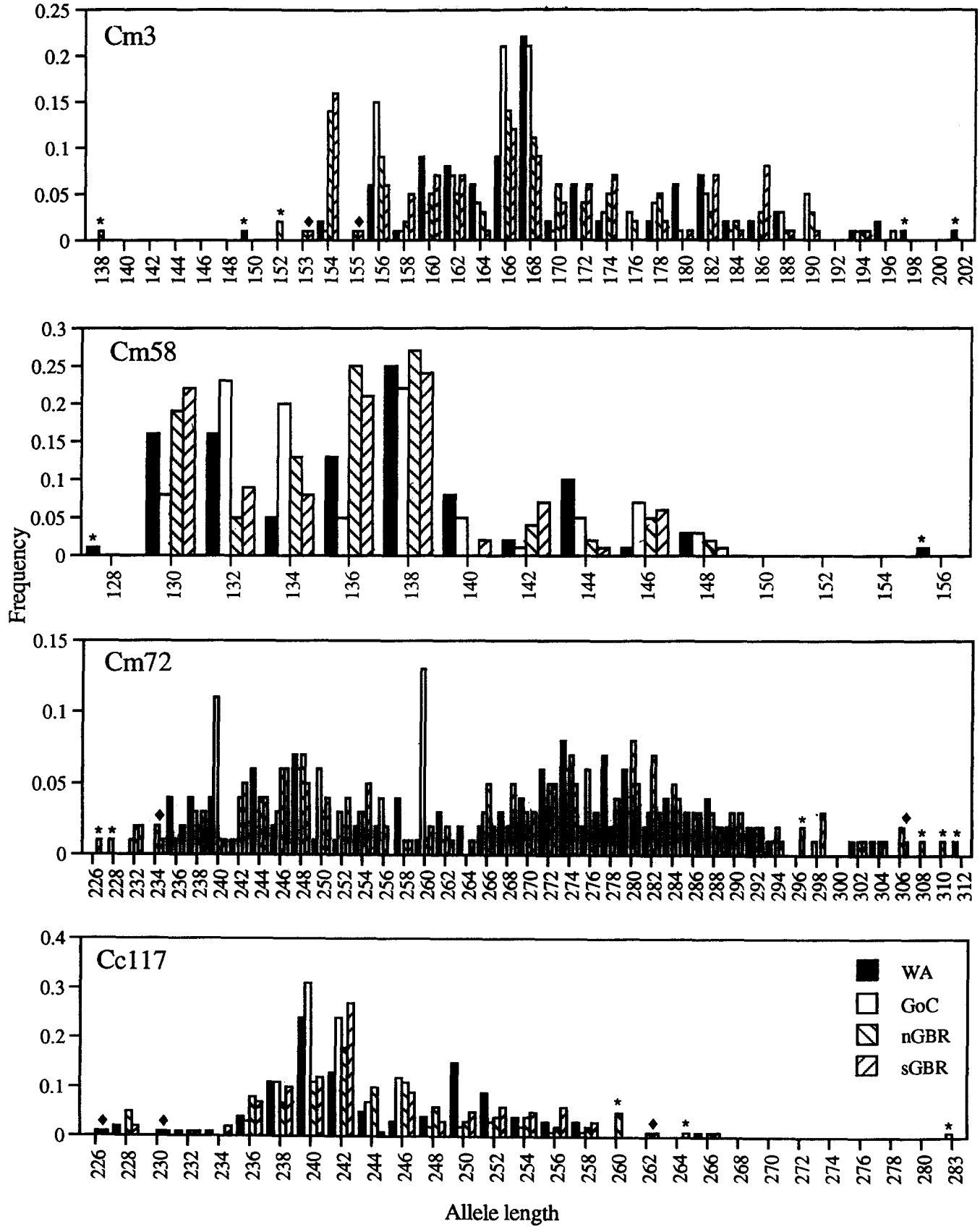

FIGURE 2.—Allele frequencies at four microsatellite loci (Cm3, Cm58, Cm72, and Cc117) in four regional populations of green turtles (*Chelonia mydas*) around the Australian coast. Populations are WA, Western Australia; GoC, Gulf of Carpentaria; nGBR and sGBR, northern and southern Great Barrier Reef. *, Unique alleles found only in particular regional populations; ●, additional alleles found only in the Pacific Ocean populations. Numeric allele frequencies are available from the authors on request.

TABLE 1

Number of alleles per locus and expected heterozygosity at microsatellite loci in regional populations of *Chelonia mydas* from Western Australia, Gulf of Carpentaria, and northern and southern Great Barrier Reef

	Location and sample size			
	WA	GoC	nGBR	sGBR
	69.9	40.3	65.8	98.0
Locus	±8.0	±7.3	±3.3	±5.4
Cm3				
A	22	18	23	22
h_{exp}	0.91	0.87	0.92	0.92
Cm58				
A	12	10	10	10
h_{exp}	0.85	0.84	0.81	0.96
Cm72				
A	33	28	35	37
h_{exp}	0.96	0.94	0.96	0.96
Cc117				
A	16	13	19	19
h_{exp}	0.88	0.81	0.91	0.88
Overall loci				
$\bar{A}$	20.8	17.3	21.8	22
H_{exp}	0.90	0.87	0.90	0.90

A, number of alleles per locus; *h* or *H*, expected heterozygosity; WA, Western Australia; GoC, Gulf of Carpentaria; nGBR, northern Great Barrier Reef; sGBR, Southern Great Barrier Reef. Sample sizes are means ± SD.

TABLE 2

Allele frequencies and observed heterozygosity at ascnDNA loci in regional populations of *Chelonia mydas* from Western Australia, Gulf of Carpentaria, northern and southern Great Barrier Reef

	Location and sample size			
	WA	GoC	nGBR	sGBR
	20.5	22.0	19.5	23.0
Locus	±0.6	±0.7	±0.3	±0.7
Cm12				
−−	0.02	0.00	0.00	0.04
−+	0.98	1.00	1.00	0.96
h	0.05	0.00	0.00	0.09
Cm14				
−−	0.02	0.00	0.00	0.00
−+	0.46	0.26	0.58	0.48
++	0.52	0.71	0.40	0.50
+[a]+	0.00	0.02	0.03	0.02
h	0.55	0.48	0.56	0.63
Cm39				
−	0.10	0.02	0.03	0.13
+	0.90	0.98	0.97	0.88
h	0.10	0.05	0.05	0.25
Cm67				
−−	0.37	0.27	0.33	0.50
−+	0.21	0.23	0.08	0.12
+−	0.42	0.50	0.60	0.38
h	0.79	0.54	0.55	0.76
Overall loci				
H	0.37	0.27	0.29	0.43

Haplotypes −, +, −−, −+, ++, and +− indicate the presence or absence of restriction sites at the following polymorphic loci: Cm12-*Alu*I and -*Hin*dII; Cm14-*Hin*fI and -*Rsa*I; Cm39-*Hin*fI; Cm45, *Alu*I and *Hin*fI; and Cm67, *Cfo*I and *Dde*I. For abbreviations, see Table 1.

[a] Denotes an additional restriction site, infrequently observed at Cm14-*Hin*fI.

observed using two of the restriction digests, Cm45 (*Hin*fI) and Cm12 (*Alu*I), previously found to be variable in green turtles on a global scale (KARL *et al.* 1992; KARL and AVISE 1993). One digestion profile, Cm45 (*Alu*I), could not be scored reliably and was dropped from the analysis. Designating individual haplotypes at multiple polymorphic sites was easily done at Cm12 and Cm14 as there were no double heterozygotes. At Cm67, double heterozygotes were scored as in KARL *et al.* (1992) as follows: given there were no ++ homozygotes observed in any samples ($n = 155$) it was assumed that double heterozygotes ($n = 8$) were all of the −+/+− type rather than −−/++. Tests for linkage disequilibrium within each region showed no significant linkage between alleles at any loci and genotype frequencies conformed to expectations of HARDY-WEINBERG equilibrium. Heterozygosity (h) was low compared to microsatellite loci and multilocus heterozygosity (H) within regions ranged from 0.27 to 0.43 (Table 2).

Genetic divergence between regions: At the microsatellite loci, significant allele frequency differences occurred between all regions except between the nGBR and sGBR (Figure 3). Allele frequencies were different ($P < 0.05$) in 19 of 24 tests between regions and most (14/24) pairwise tests remained significant after corrections for multiple tests (12 pairwise tests per region). In general, less divergence in allele frequencies was observed at the Cm72 locus relative to the other three loci, suggesting that the greater number of alleles at this locus decreased the power to detect divergence at regional levels of gene flow. In contrast to the microsatellite data, there were few significant differences in allele frequency at ascnDNA between regions (3/24 tests), and none of these remained significant when corrected for multiple tests (Figure 3). Among all regions combined, allele frequencies were strongly differentiated at each microsatellite locus ($P < 0.0005$) whereas allele frequencies were not different at ascnDNA loci ($P > 0.05$).

Measures of genetic structure: Nested analyses of molecular variance (AMOVA; EXCOFFIER *et al.* 1992) at microsatellite loci indicated that 98.5–99.9% of the genetic variation was contained within rookeries, such that <1% of the variation occurred among rookeries within regions, or among regions. In contrast, only 22.5% of the genetic variation in mtDNA haplotypes occurred within rookeries, whereas 77.5% was partitioned among regions and none among rookeries within regions.

Among regions, F_{st} estimates were low at each microsatellite locus (0.002–0.019) but significantly different from zero, and across all loci combined, $F_{st} = 0.014$ (P

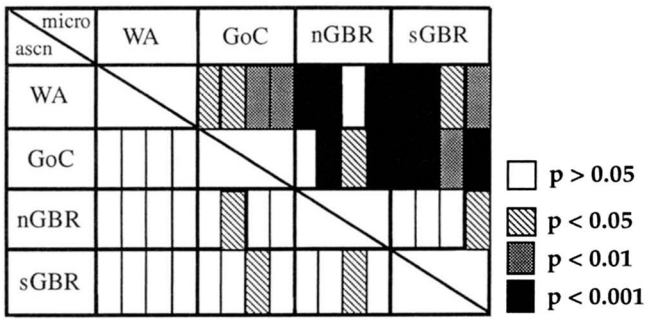

FIGURE 3.—Pairwise tests of allele frequency differences at microsatellite loci (above diagonal) and ascnDNA loci (below diagonal) between regional populations of green turtles (*Chelonia mydas*). Probability values were determined from randomized data sets generated in REAP (McELROY *et al.* 1992) and are not corrected for multiple tests. For each comparison between regions, from left to right, the four microsatellite loci are Cm3, Cm58, Cm72, and Cc117 and the four ascnDNA loci are Cm12, Cm14, Cm39, and Cm67. Populations are WA, Western Australia; GoC, Gulf of Carpentaria; nGBR and sGBR, northern and southern Great Barrier Reef.

TABLE 3

Measures of genetic subdivision among four regional populations of *Chelonia mydas* from Western Australia, Gulf of Carpentaria, and northern and southern Great Barrier Reef

Microsatellite loci			ascnDNA loci	
Locus	$F_{st}(\theta)$	R_{st}	Locus	$F_{st}(\theta)$
Cm3	0.014***	0.014, NS	Cm12	0.000, NS
Cm58	0.015***	−0.006, NS	Cm14	0.041*
Cm72	0.002*	0.017, NS	Cm39	0.015, NS
Cc117	0.019***	0.002, NS	Cm67	0.018, NS
Overall	0.014***	0.007*	Overall	0.027**

R_{st} was calculated using R_{st}CALC (GOODMAN 1997) and levels of significance were derived from permutation testing of R_{st} analogues in AMOVA (MICHALAKIS and EXCOFFIER 1996) under a model of squared base pair length differences between alleles (see text). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. NS, not significant.

< 0.001; Table 3). In comparison, R_{st} over all loci was lower and had a decreased level of significance (R_{st} = 0.007, $P < 0.05$; Table 3). R_{st} was greater than F_{st} only at the locus with the most alleles (Cm72; Table 3). At ascnDNA loci, the overall F_{st} among regions was low (F_{st} = 0.027) but significantly different from zero (P = 0.007) with a level of significance that was intermediate to microsatellites under the IAM (F_{st}) or SMM (R_{st}) measures (Table 3). Estimated F_{st} for the mtDNA haplotypes indicated a substantially greater degree of genetic subdivision (F_{st} = 0.82) than indicated for either of the nuclear marker sets.

In pairwise tests between regions, F_{st} values for microsatellite loci were significantly greater than zero in all comparisons ($P < 0.001$) except between the nGBR and sGBR populations (Table 4). In contrast, R_{st} values indicated little genetic differentiation between regions and only two comparisons (WA-GoC and GoC-nGBR) showed significant heterogeneity ($P < 0.05$; Table 4). In these tests, R_{st} values varied considerably more than F_{st} values, the two sets of values were poorly correlated (r^2 = 0.23, d.f. = 5, P = 0.34), and R_{st} showed less agreement with geographic scale. At the ascnDNA loci, significant heterogeneity was indicated only between the GoC-nGBR and the GoC-sGBR populations ($P <$ 0.01; Table 4). In contrast to microsatellite F_{st} values, ascnDNA-derived F_{st} estimates were not significantly different from zero in any of the three comparisons involving the WA population and the lowest divergence was observed between the geographically most distant WA and sGBR populations. In several cases, the F_{st} value at ascnDNA loci was substantially greater than that for microsatellites but had much greater variation (see standard deviations in Table 4) among loci and lower significance. In contrast to F_{st} and R_{st} values for nuclear markers, pairwise F_{st} values for mtDNA haplotypes indicated a high level of genetic heterogeneity between all regions, including the nGBR and sGBR populations ($P < 0.001$; Table 4). These high F_{st} values for mtDNA are consistent with results of pairwise chi-square tests ($P < 0.001$) between the same regions in NORMAN *et al.* (1994).

Estimated rates of gene flow: Estimates of gene flow, which assume migration-drift equilibrium (SLATKIN 1987), varied considerably between different measures for microsatellites, different classes of nuclear loci, and nuclear loci *vs.* mtDNA (Figure 4). For microsatellite data, estimates from F_{st} and R_{st} were similar, except for comparisons between the GoC and neighboring nGBR and WA regions where the R_{st}-based estimates were lower. Regardless, all F_{st}- and R_{st}-based values were substantially greater than Nm = 1.0 and the highest level of inferred gene flow was between nGBR and sGBR. Estimates of migration rate inferred from ascnDNA were similar to the IAM and SMM estimates for microsatellites for the WA-GoC and WA-nGBR comparisons, but otherwise showed little consistency and, overall, were poorly correlated to either ($r^2 \leq 0.04$, $P \geq 0.72$). Two notable deviations were between nGBR and sGBR, where microsatellites indicated extensive gene flow but ascnDNA loci suggested moderate gene flow, and between WA and sGBR, the two most distant regions, where the ascnDNA loci indicated extensive gene flow but microsatellites suggested moderate gene flow.

Estimates of female-based gene flow were typically very low ($Nm_f \ll 1.0$), the exception being between the two Indian Ocean regions, GoC and WA, where Nm_f = 1.4. Even allowing for a twofold difference (*i.e.*, assuming that $N_f = 0.5\ N_e$) expected if rates are the same between males and females (*i.e.*, $m_f = m$), migration rates estimated from mtDNA are mostly an order of magnitude less than those from nuclear genes. The difference is particularly striking for the comparison

TABLE 4

Measures of population subdivision for microsatellite loci, ascnDNA loci, and mtDNA control region haplotypes between regional populations of *Chelonia mydas* from Western Australia, Gulf of Carpentaria, and northern and southern Great Barrier Reef

	Microsatellites		ascnDNA	mtDNA
Comparison	$F_{st}(\theta)$	R_{st}	$F_{st}(\theta)$	$F_{st}(\theta)$
WA				
GoC	0.012 ± 0.001***	0.023 ± 0.002*	0.014 ± 0.031, NS	0.26***
nGBR	0.018 ± 0.006***	0.0081 ± 0.001, NS	0.014 ± 0.007, NS	0.82***
sGBR	0.017 ± 0.005***	0.0075 ± 0.0008, NS	−0.0080 ± 0.009, NS	0.87***
GoC				
nGBR	0.022 ± 0.008***	0.021 ± 0.002*	0.074 ± 0.085**	0.65***
sGBR	0.024 ± 0.006***	0.0081 ± 0.001, NS	0.052 ± 0.014**	0.76***
nGBR				
sGBR	0.0010 ± 0.002, NS	−0.0033 ± 0.0005, NS	0.027 ± 0.022, NS	0.83***

See text for calculations of F_{st} and R_{st}. Western Australia comparisons exclude Sandy Island. For abbreviations, see Table 1. Values are means ± SD. NS, not significant. P values as in Table 3.

between the nGBR and sGBR regions, where the microsatellite analyses indicated levels of gene flow two to three orders of magnitude greater than for mtDNA.

DISCUSSION

Comparison of population structure at nuclear loci: ascnDNA loci typically indicated less regional heterogeneity in green turtle populations than microsatellite loci. Differences in allele frequencies among the four regions combined were nonsignificant at each of the four ascnDNA loci, but significant at each of the four microsatellite loci. In pairwise tests, significant heterogeneity was observed for three of 24 comparisons of ascnDNA loci in comparison to 19 of 24 tests of microsatellite loci (Figure 3). The difference in sensitivity was also apparent from the number of region-specific alleles; only one was observed for the ascnDNA loci (Cm14, in WA), whereas 16 were observed at microsatellite loci. This could, to some extent, reflect sampling effects as the population-specific microsatellite alleles were observed at low frequencies (<0.05) and the number of alleles detected was positively correlated with sample size. Regardless, the observation that most unique alleles at microsatellite loci (Figure 2) were at or near the extremes of the allele size distributions suggests that these locality-specific alleles represent mutations that have arisen within regions and have not yet spread by migration (SLATKIN 1995a). Selection against the larger unique alleles (GARZA *et al.* 1995) may limit their occurrence and contribute to their usefulness as population markers, although in the present case there were as many region-specific alleles in both the large (n = 12) and small (n = 11) size range.

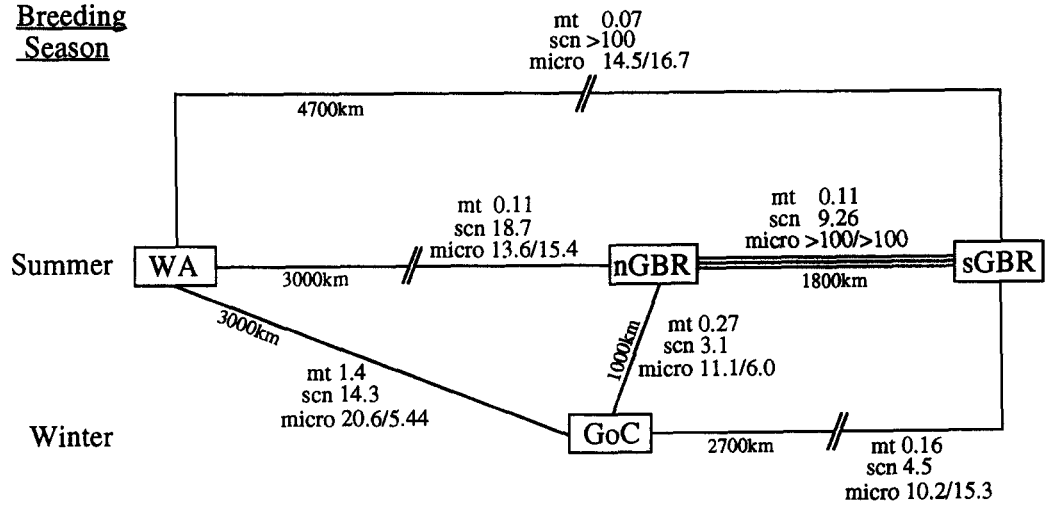

FIGURE 4.—Estimates of gene flow (Nf_m and Nm) between regional populations of green turtles (*Chelonia mydas*) based on F_{st} values for mtDNA (mt), and ascnDNA (ascn), as well as F_{st} and R_{st} values for microsatellites (micro F_{st}/R_{st}). The approximate distance between regional nesting beaches and the timing of regional breeding seasons are indicated. Populations are WA, Western Australia; GoC, Gulf of Carpentaria; nGBR and sGBR. northern and southern Great Barrier Reef. For microsatellites (F_{st}) all pairwise comparisons are significant, except for between nGBR and sGBR (indicated by triple horizontal lines).

The overall proportion of variation distributed among regions at ascnDNA loci (F_{st} = 0.027 ± 0.012) was greater than either the IAM (F_{st} = 0.014 ± 0.004) or SMM (R_{st} = 0.0067 ± 0.0004) estimates for microsatellite loci, but with an intermediate level of significance due to greater variance among ascnDNA loci (Table 3). Several of the results from the ascnDNA loci appear anomalous in the context of the geographic distribution of regions, including the lack of divergence between WA and any other region and the high rate of gene flow indicated between WA and sGBR, the most distant breeding populations (Figure 4). By contrast, the microsatellite data appear more compatible with predictions from tagging data regarding the potential for gene flow between regions. We suggest, therefore, that the microsatellite loci provide a more accurate picture of nuclear gene variation among Australian populations than do the ascnDNA loci. The proportion of variance observed among Australian populations at ascnDNA loci is, however, substantially lower than that observed by KARL *et al.* (1992) on a global scale (F_{st} = 0.17) or among more distant rookeries from the Indo-Pacific (F_{st} = 0.13) and it remains to be determined whether microsatellites or ascnDNA perform better at this broader geographic scale.

The larger variance among ascnDNA loci is of interest given recent evidence suggesting qualitative differences in population structure of oysters as inferred from ascnDNA loci isolated from different laboratories (KARL and AVISE 1992; MCDONALD *et al.* 1996). One possible explanation for this large variance is that variation at individual ascnDNA loci is strongly affected by linkage to other loci subject to selection (BEGUN and AQUADRO 1992; CHARLESWORTH *et al.* 1993). Although microsatellite loci may also be affected by this phenomenon (SLATKIN 1995b), variation within and among populations should be restored more rapidly because of the higher mutation rate.

Assuming that microsatellites are providing a more accurate interpretation of population structure, it remains to be determined which mutation model should be used, as evidence on mutational processes from other species is conflicting (DI RIENZO *et al.* 1994; ESTOUP *et al.* 1995; GARZA *et al.* 1995; SHRIVER *et al.* 1995). Several observations suggest that, for green turtles, the SMM, and thus R_{st}, is theoretically more appropriate than the IAM, but that high migration rates moderate its effectiveness as a predictor of gene flow. (1) Preliminary data on mutations (N. FITZSIMMONS, unpublished results) indicate that preexisting alleles are regenerated (contra the IAM assumption). However, as only a minority of inferred mutations (~30%, FITZSIMMONS, unpublished results) were of a single step, the *strict* SMM appears inappropriate. (2) Nearly all possible allele sizes are observed within the size range, and locality-specific alleles are largely restricted to the extremes of the size distribution (Figure 2). This suggests that, as alleles are being lost to drift, they are replaced by mutations to the same size, and even mutations of large effect may arise undetected. (3) In comparisons between adjacent regions, Nm estimates from R_{st} are lower than those from F_{st} for moderate gene flow rates (*i.e.*, between GoC and the adjacent nGBR and WA regions), but not where high gene flow is inferred (nGBR-sGBR; Figure 4). These trends are consistent with the results of SMM simulations (SLATKIN 1995a), despite the high proportion of multistep mutations at these loci and the relatively recent founding of the GoC rookery (<9500 years; JONES and TORGERSEN 1988). However, paradoxically, fewer of the R_{st} values were significant in pairwise tests, and most of the values were lower than F_{st}. This is not a consequence of different methods for testing as both were based on similar permutation procedures.

The performance of R_{st} *vs.* F_{st} is also expected to improve as mutation rate increases (SLATKIN 1995a). This was apparent in our data in that the locus with largest number of alleles and the highest mutation rate (Cm72; N. FITZSIMMONS, unpublished results) showed the greatest increase of R_{st} over F_{st} among all regions combined (R_{st} = 0.017, F_{st} = 0.002). By contrast, R_{st} was similar to or lower than F_{st} at loci with fewer alleles, the extreme being the least variable locus (Cm58; R_{st} = −0.006, F_{st} = 0.015). At all loci other than Cm72, it appears that the geographic distribution of variation is dominated by migration and drift, rather than the accumulation of site-specific mutations. Variation in performance was also found between F_{st} estimates and several measures of R_{st} in humpback whales (VALSECCHI *et al.* 1997). It remains to be ascertained how much of the variation in results is due to the dynamics of microsatellite mutations, including constraints on allele size (GARZA *et al.* 1995) and size homoplasy (ESTOUP *et al.* 1995) that lead to alleles of same size not being identical by descent and violate assumptions inherent in the various models. These phenomena may cause substantial interlocus variation in measures of genetic subdivision, leading to underestimates of divergence among populations at some loci.

Nuclear *vs.* mitochondrial gene flow in green turtles: Similar to the comparison of ascnDNA and mtDNA among global populations (KARL *et al.* 1992), levels of gene flow at nuclear loci were substantially greater than that for mtDNA among Australian populations. However, in contrast to the previous data, genetic heterogeneity was observed between nearly all regions at microsatellite loci, with the important exception of the nGBR and sGBR populations. Excluding this anomaly, tests for heterogeneity of allele frequencies revealed significant differences between, but not within, regions for both mtDNA (NORMAN *et al.* 1994) and microsatellites. The nGBR-sGBR comparison is exceptional in that mtDNA shows a substantial (90%) difference in the frequency of the two dominant alleles, yet none of the microsatellite loci were statistically heterogeneous and, overall, all measures of among-population variance at nuclear loci were nonsignificant.

Estimates of equilibrium migration rates from nuclear genes varied depending on the measure used and the type of locus analyzed, but these were generally much greater than 1.0 whereas mtDNA estimates were usually much less than 1.0. Given that the Nm estimates correct for haploid, but not maternal inheritance, the expected difference is twofold (*i.e.*, $N_f m_f$ *vs.* $N_e m$) if there is no sex-biased gene flow ($m_f = m$). Excluding the nGBR-sGBR comparison, average Nm estimates from microsatellites using F_{st} and R_{st} measures are 45 and 50 times greater than for mtDNA, respectively (Figure 4). These observations suggest that there is sufficient male-mediated gene flow to limit the extent of regional isolation without creating panmixia. An alternative explanation is that the difference in partitioning of nuclear and mitochondrial DNA variation is due to recent separation of populations and a slower approach to migration-drift equilibrium for nuclear genes (BIRKY *et al.* 1989). This is not supported because the GoC and WA populations, which mtDNA phylogeography suggests are the most recently separated (NORMAN *et al.* 1994; C. MORITZ, C. LIMPUS and R. SLADE, unpublished results) show the lowest ratio of nuclear:mitochondrial divergence (Figure 4). The most dramatic difference is between the nGBR and sGBR where estimates of nuclear gene flow were over 1000 times greater than for mtDNA and the populations are effectively panmictic for nuclear genes.

The evidence for greater levels of nuclear than mitochondrial gene flow across all populations is consistent with the global pattern reported by KARL *et al.* (1992), although we found that ascnDNA (as used by KARL *et al.* 1992) had less power and precision than microsatellites to detect population subdivision. KARL *et al.* (1992) suggested several mechanisms for male-mediated gene flow, including reduced philopatry by males and mating of females in feeding grounds or along migratory routes shared by otherwise separate breeding populations. Comparison of mtDNA markers in nesting females and courting males from the GoC, nGBR, and sGBR rookeries indicate that males are as philopatric as females, excluding this explanation for the Australian green turtle populations (FITZSIMMONS *et al.* 1997).

Interpopulation mating at feeding grounds or during migration depends on the timing and duration of mating behavior, which may vary between sexes and geographically. Males are reproductively active for ~2 mo (LICHT *et al.* 1985; LIMPUS 1993) but breeding females may be receptive for a much shorter period (<2 wk in captivity; COMUZZIE and OWENS 1990). Whether unreceptive females can prevent insemination by courting males is unknown, but successful avoidance of courtship has been observed in various circumstances (BOOTH and PETERS 1972; COMUZZIE and OWENS 1990). Most known mating occurs just prior to the onset of the nesting season in established courtship areas, and females store sperm to fertilize successive clutches (OWENS 1980; N. FITZSIMMONS unpublished results). These observations suggest that matings between regional populations is unlikely to occur on joint feeding grounds prior to migration and that attention should be directed to the potential for nuclear gene flow due to matings by both males and females during their migration.

The combined genetic and tagging evidence for Australian populations support the hypothesis that nuclear gene flow is primarily mediated by matings between turtles from different regional populations during the migrations from feeding grounds to courtship areas. The least discrepancy between mitochondrial and nuclear gene flow rates was observed between the GoC and adjacent nGBR and WA regions. This is significant because the GoC population primarily breeds in winter (due to sand temperatures in the summer being too high for embryonic development), whereas the other populations breed in summer (Figure 4). This would reduce the potential for matings between GoC and adjacent rookeries during breeding migrations. In contrast, the greatest excess of nuclear over mitochondrial gene flow occurs between the nGBR and sGBR rookeries. To reach their own breeding sites, those sGBR females (and males; FITZSIMMONS *et al.* 1997) resident in the Torres Strait and further to the west must migrate through the major nGBR courtship area (Figure 1) at the same time as the nGBR population is reproductively active. This creates the potential for matings of sGBR females by nGBR males and nGBR females by sGBR males, leading to nuclear, but not mitochondrial gene flow. This hypothesized mechanism for nuclear gene flow can, in principle, be tested for turtles mating in Torres Strait through (1) mark-recapture studies, (2) identification of known sGBR turtles with fresh courtship damage in Torres Strait, or (3) by comparisons of mtDNA haplotype frequencies of courting turtles to those of the nGBR nesting population.

If, as we suggest, nuclear gene flow in green turtles is due to interregional matings during migrations, especially by turtles that pass through courtship areas of other regional populations, then the difference in geographic structure of nuclear *vs.* mitochondrial polymorphism may represent differential gene flow that occurs without differences between sexes in either dispersal or assortative mating. In other words, males may have the same natal philopatry as females, but interpopulation gene flow results from variation in the timing of migration or opportunistic matings during migration, largely a consequence of the location of courtship areas relative to feeding grounds. It can only be referred to as male-mediated gene flow in the sense that sperm are exchanged among regional populations, whereas eggs are not. Whether this mechanism can account for the global pattern depends on the opportunities for mating during migrations, which can only be assessed by combining detailed ecological evidence on migration pathways with the genetic patterns. More generally, although male-biased gene flow is commonly invoked where mtDNA (maternal) gene flow appears lower than

nuclear (biparental) gene flow (*e.g.*, PALUMBI and BAKER 1994; MELNICK and HOELZER 1992), the case for Australian green turtles highlights the value of incorporating behavioral, ecological, and genetic data to investigate further the cause of such differences.

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