

The genetic structure of Australasian green turtles (*Chelonia mydas*): exploring the geographical scale of genetic exchange

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Abstract

Ecological and genetic studies of marine turtles generally support the hypothesis of natal homing, but leave open the question of the geographical scale of genetic exchange and the capacity of turtles to shift breeding sites. Here we combine analyses of mitochondrial DNA (mtDNA) variation and recapture data to assess the geographical scale of individual breeding populations and the distribution of such populations through Australasia. We conducted multiscale assessments of mtDNA variation among 714 samples from 27 green turtle rookeries and of adult female dispersal among nesting sites in eastern Australia. Many of these rookeries are on shelves that were flooded by rising sea levels less than 10 000 years (*c.* 450 generations) ago. Analyses of sequence variation among the mtDNA control region revealed 25 haplotypes, and their frequency distributions indicated 17 genetically distinct breeding stocks (Management Units) consisting either of individual rookeries or groups of rookeries in general that are separated by more than 500 km. The population structure inferred from mtDNA was consistent with the scale of movements observed in long-term mark–recapture studies of east Australian rookeries. Phylogenetic analysis of the haplotypes revealed five clades with significant partitioning of sequence diversity ($\Phi = 68.4$) between Pacific Ocean and Southeast Asian/Indian Ocean rookeries. Isolation by distance was indicated for rookeries separated by up to 2000 km but explained only 12% of the genetic structure. The emerging general picture is one of dynamic population structure influenced by the capacity of females to relocate among proximal breeding sites, although this may be conditional on large population sizes as existed historically across this region.

Keywords: effective population size, Indo-Pacific, Management Units, mtDNA, phylogeography, testudines

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Introduction

Green turtles (*Chelonia mydas*) are long-lived, herbivorous reptiles with a circumtropical distribution. Understanding the complex life history of this species has been a major challenge to biologists because of the spatial and temporal

scales involved. Knowledge of population dynamics is largely obtained from long-term mark–recapture studies of females tagged while nesting on the beaches. These studies show that breeding female turtles display high fidelity to the same nesting beaches (Hendrickson 1958; Carr & Ogren 1960) and Carr (1967) hypothesized that mature nesting female turtles were selecting their natal beach to deposit eggs. Studies in the southern Great Barrier Reef (sGBR) demonstrate that green turtles also display fidelity to resident feeding grounds throughout their adult lives (Limpus *et al.* 1992) with females reaching sexual maturity at about 40 years (Limpus & Chaloupka 1997). Recaptures of tagged turtles on distant feeding grounds provided evidence that at least a proportion of the green turtle population migrates over geographically large distances between nesting and feeding habitat (Dizon & Balazs 1982; Meylan 1982; Limpus 1992), and this is corroborated by satellite tracking data (e.g. Papi *et al.* 1995; Craig *et al.* 2004). In spite of the nesting site fidelity, female green turtles have been observed to deposit clutches on different nesting beaches within and among breeding seasons (e.g. Limpus *et al.* 2003), but the geographical scale over which females switch nesting beaches remains unclear.

Analysis of mitochondrial DNA (mtDNA) structure in Atlantic green turtle populations supported the natal homing hypothesis, as geographically distant rookeries were found to have heterogeneous mtDNA haplotype frequencies (Bowen & Avise 1996). Similarly Norman *et al.* (1994) found significant geographical structuring of mtDNA variation among eight of nine widespread rookeries studied in the Australasian region and FitzSimmons *et al.* (1997a) inferred that male green turtles, like females, are philopatric to natal regions. The observed heterogeneity of mtDNA haplotypes among regional rookeries indicates the presence of multiple distinct genetic stocks (Bowen *et al.* 1992; Norman *et al.* 1994; Encalada *et al.* 1996) or ‘management units’ (MUs; Moritz 1994). An understanding of female movements as provided by mtDNA is of particular relevance to defining such MUs because colonization by females is crucial to maintaining viability of metapopulations following disturbance (Avise 1995). One limitation common to all of the above mtDNA studies is that they rarely compare closely spaced (< 500 km) rookeries, so that the extent of exchange on a local scale has not been determined. A second general limitation of such studies is that, despite the emphasis placed on mtDNA differentiation (e.g. Moritz 1994; Avise 1995), there is considerable uncertainty in estimating migration rates from mtDNA (Whitlock & McCauley 1999; Ballard & Whitlock 2004). In this context, it is highly desirable to combine mtDNA evidence with mark–recapture data to test for congruence. Other than broad-scale comparisons (e.g. Meylan *et al.* 1990), this has not yet been done for marine turtles. Thus, despite decades of tagging and genetic studies, we do not yet know the geographical scale of an individual

breeding population; is it a single beach, immediately adjacent beaches, or a whole archipelago? Resolution of this question is essential to understanding how green turtles respond to changes in the availability of nesting sites over time, for example when nesting beaches are lost due to increasing sea levels as a result of climate change.

For green turtles, comprehensive distribution and movement patterns are being elucidated by tag–return data, but information from these studies is skewed by tagging and survey effort and typically applies only to adult females. Although the sGBR green turtle population appears to be increasing (Chaloupka & Limpus 2001), populations throughout much of the Australasian region are declining (Sloan *et al.* 1994; Limpus 1997). This has been attributed to various causes including the harvest of eggs and turtles for food, by-catch in fisheries activities, loss of nesting habitat and diseases such as fibropapillomas (Limpus & Parmenter 1985; Broderick 1998; Kennett *et al.* 1998; Dethmers 2000). In Pangumbahan (Java) and Berau (east Kalimantan), the dramatic decrease in egg production as a result of extended egg concession practices is well documented (Wicaksono 1992; Sloan *et al.* 1994; Limpus 1997). More dramatic is the situation on Bali where a complete loss of all green turtle rookeries has resulted from local overharvesting of eggs and turtles (Schulz 1984). The precipitous decline of some green turtle populations over the past few centuries (Limpus 1995) has increased the need to detect geographical boundaries and identify demographically independent populations for management.

The present study examines the mtDNA variation across 27 green turtle rookeries in Australasia (western Pacific Ocean, eastern Indian Ocean, and the Southeast Asian seas), representing all major breeding sites in the region. To test the assumption that variation in mtDNA haplotype frequencies predicts low rates of exchange, we compare genetic inferences against available recapture data, especially for the intensively studied east Australian populations. The Australasian region is especially relevant to examining potential for relocation of rookeries following sea level change as many of the sites studied are on shallow platforms that were only available to marine turtles from < 10 000 years (c. 450 generations) ago (Fig. 1; Chappell & Shackleton 1986; Torgersen *et al.* 1985). Sea level changes associated with glacial cycles have repeatedly exposed and flooded vast areas on the Sunda and Sahul shelves approximately every 100 000 years (Chappell & Shackleton 1986; Torgersen *et al.* 1985). As a result, nesting habitat was created or removed. This would have resulted in repeated and large shifts of populations as gravid female turtles were forced to find new suitable nesting habitat. From patterns of sequence variation in mtDNA and available recapture data we infer population boundaries among rookeries and thereby identify MUs for Australasian green turtles to facilitate national and international management and as



Fig. 1 Rookeries sampled (black circles) for this study and distribution of green turtle rookeries throughout the Australasian region (grey circles). Estimated annual number of females at each of the rookeries is proportional to the size of the circles as indicated in the legend. Rookery size data have been derived from the Marine Turtle Interactive Mapping System (UNEP/CMS www.unep-wcmc.org) and the Marine Turtle Database maintained by C. J. Limpus at Queensland Parks and Wildlife Service. Shaded area denotes approximate landmass contours at sea levels 120 m below current levels.

baseline data for subsequent analyses of foraging grounds and harvests. We also examine large-scale patterns of sequence diversity across the Indo-Pacific divide in the context of historical shifts in the location of nesting habitats.

Materials and methods

Sampling was designed to cover all of the known major and historically important rookeries ($n = 27$) throughout Southeast Asia, Australia, the western Pacific and eastern Indian Oceans (Table 1 and Fig. 1). For several regions [southern and northern Great Barrier Reef (sGBR and nGBR), Gulf of Carpentaria (GoC), Sulu Sea and South China Sea; Table 1] we were able to sample turtles from multiple adjacent rookeries. This provided a range in geographical distances among sampled rookeries from 14 to 7799 km and a total sample size of 714 individuals. Sample sizes varied extensively (9–60 per rookery) due to nesting population size and logistic constraints. DNA was extracted from skin tissue or blood from nesting females or

from hatchlings (including samples used in Norman *et al.* 1994 and FitzSimmons *et al.* 1997b), ensuring that progeny from a given female were only sampled once. Skin biopsies were stored in a NaCl saturated solution of 20% DMSO and blood cells were either frozen or suspended in a long-term storage buffer (100 mM Tris, 100 mM NaCl, 10 mM EDTA.2Na, 0.5% SDS).

Over the past three decades many thousands of green turtles along the east coast of Australia received self-piercing, self-locking tags initially made of monel and later of titanium, as part of a long-term mark–recapture study (Limpus 1992). Tagging data from the 1998–1999 nesting season were analysed to estimate movement patterns of nesting females among rookeries. Distances between rookeries in the nGBR range from 5 to 460 km and in the sGBR from 12 to 442 km. There is only rare nesting throughout the 620-km stretch of the Great Barrier Reef that separates the southern most rookery in the nGBR (no. 7 Sandbank, unsampled) and the northern most rookery in the sGBR (Cockermouth and Bushy Islands, unsampled) (Fig. 1).

DNA was extracted from small amounts of tissue (typically 0.1 g) or blood (~10 µL) and prepared for polymerase chain reaction (PCR) as described in Norman *et al.* (1994). A 384-bp segment of the mtDNA control region was amplified using TCR5 and TCR6GC primers (modified after Norman *et al.* 1994; with the latter primer containing a 41-bp GC clamp). Typically, 1–2 µL of template was used in 25-µL PCR reactions using standardized conditions of denaturing at 94 °C for 10 s, annealing at 56 °C for 30 s and extension at 72 °C for 40 s for 32 cycles.

To process the large number of samples, we developed a rapid yet sensitive screening protocol using denaturing gradient gel electrophoresis (DGGE; Myers *et al.* 1987) to detect DNA polymorphisms. Polymorphism was detected after 14 h of electrophoresis at 80 V and 58 °C through a 1×TAE 6.5% polyacrylamide gel whose denaturant gradient ranged from 30% to 45% (32% formamide and 5.6 M urea). Sensitivity was increased by hybridizing candidate DNA variants with known sequence variants of similar mobilities to differentiate homo- vs. hetero-duplexes. A mixture of equal quantities (2.5 µL) of candidate and known sequence variants was denatured at 90 °C for 5 min., followed by 10 min gradual cooling from 70 °C to 50 °C. For one group of sequence variants with a similar melting behaviour we used outgroup heteroduplexing (Campbell *et al.* 1995). The gels were silver stained, and archived. The screening strategy we employed was to (i) score samples relative to the homoduplex mobility of all known mtDNA haplotypes, (ii) confirm their identity using heteroduplex analysis, and (iii) sequence representatives from each genotype/locality combination for final verification and to test sensitivity.

Sequencing was facilitated by the use of M13 forward (GAGCGGATAACAATTTCACACAGG) and reverse (AGGGTTTCCAGTCACGACGTT) universal primers that annealed to complementary tails that were added to the TCR5 and TCR6 (without the GC-clamp) primers. Dye terminator cycle sequencing was done in 10-µL reactions with 1.6 µM of the M13 primer and 30 cycles of denaturing at 96 °C for 20 s, annealing at 50 °C for 20 s and extension at 60 °C for 4 min on an ABI 3730 automated sequencer. Each specimen was sequenced in both directions.

Sequences were aligned using CLUSTAL X (Thompson *et al.* 1997) and population genetic parameters estimated in ARLEQUIN 2.000 (Schneider *et al.* 2000). Estimates of nucleotide (π) and haplotype (h) diversity, pairwise F_{ST} tests (10 000 replicates; Slatkin 1991), exact tests of population differentiation (100 000 replicates; Raymond & Rousset 1995) and AMOVA (10 000 replicates; Excoffier *et al.* 1992) were used to quantify genetic diversity. In the AMOVA, rookeries were grouped by their identified management units (stocks) and by ocean basin. Both sequence-based (Φ_{ST}) and conventional F_{ST} distance measures were used to calculate within- and among-population diversity.

The program MODELTEST 3.7 (Posada & Crandall 1998)

was used to choose among models of sequence evolution that best fit our data. The data set considered in this paper is a subset of the global diversity of sequence variants described in *Chelonia mydas* (D. Broderick, unpublished). We therefore used our data in combination with a global data set of *C. mydas* sequence variants (selected from GenBank; Broderick, unpublished data) to determine the most likely of 56 substitution models. Heuristic maximum-likelihood and a maximum-parsimony analysis using a tree-bisection-reconnection (TBR) branch-swapping algorithm with 200 and 1000 bootstrap replicates, respectively, were used to test the robustness of the inferred phylogeny. Phylogenetic analysis were performed using the program PAUP* 4.10b (Swofford 2001). Parsimony haplotype networks were estimated in ARLEQUIN 2.000 and graphically represented with the assistance of the TCS program (Clement *et al.* 2000).

Isolation by distance (IBD), under a two-dimensional stepping-stone model, was tested in GENALEX (Peakall & Smouse 2001) using conventional regression analysis [natural log (ln) of sea distance (km) vs. genetic differentiation ($F_{ST}/1 - F_{ST}$); Rousset 1997] and the statistical significance of the correlation was tested using Mantel's test (5000 iterations; Mantel 1967). Patterns of spatial genetic autocorrelation were examined over increasing distance classes and significant departures from spatially random distributions were detected by permutation 1000 replicates in GENALEX. A computational geometry approach as implemented in Barrier (Manni *et al.* 2004) was used to further explore the spatial genetic landscape and identify areas where genetic barriers between adjacent rookeries might be located. Shortest sea distance between rookeries was calculated using the great circle distance that incorporates the curvature of the earth. We refrained from use of coalescent methods to estimate population parameters (e.g. theta, growth, migration) as exploratory analyses revealed very unstable results, as expected for single locus estimates (Kuhner *et al.* 1998).

Following the simple approach of Lahanas *et al.* (1994), long-term, female effective population sizes (N_{ef}) were compared with current effective population sizes (N_{af} estimates of annual numbers of breeding females) in each MU. N_{ef} was estimated using the relationship $N_{ef} = \pi/2\mu$, where μ is the mutation rate per generation. A range in mutation rates from 0.006 to 0.012 substitutions per site per million years was taken from Encalada *et al.* (1996). We expressed N_{ef} in number of turtles per year by maintaining a generation time of 40 years as estimated for green turtles nesting along the east coast of Australia (Limpus & Chaloupka 1997) and an average interbreeding period of 5.5 years (Limpus *et al.* 1994). N_{af} was derived from the Marine Turtle Interactive Mapping System (UNEP/CMS www.unep-wcmc.org) and the Marine Turtle Database maintained by C. J. Limpus. We used the sum of median values of population estimates where multiple rookeries occurred within a MU.

Results

Haplotype identification using DGGE

Of the 25 distinct haplotypes identified via direct sequencing, only three sets of haplotypes could not be resolved using routine heteroduplexing. Haplotypes C1, C3, and C14 were subsequently distinguished using outgroup heteroduplexing (Campbell *et al.* 1995) with the C12 haplotype, providing distinct heteroduplex band patterns. Haplotypes C4 and C8 had identical melting behaviour under all conditions and were distinguished using the restriction enzyme *Sau96I*. Haplotypes E1 and E2 differed by a single base pair (bp) but were indistinguishable by heteroduplexing and were grouped together as the E1/E2 haplotype in further analyses. Sequencing of representatives of all haplotypes from all populations revealed no additional sequence variants. This confirmed that our DGGE assays provided a highly sensitive, repeatable and cost-effective strategy to rapidly screen for variants in marine turtle populations. It is important to note that this work was carried out when sequencing was still very expensive for us and that the same methods were used to screen mtDNA variants in thousands of samples from feeding grounds and harvests (K. Dethmers & D. Broderick, unpublished data).

The best-fit model of sequence evolution as selected by the hierarchical likelihood-ratio tests (hLRTs) was the Hasegawa–Kishino–Yano model (Hasegawa *et al.* 1985) (HKY + G + I). The second best-fit model was Tamura–Nei (Tamura & Nei 1993) (TrN), with G = 0.25 and I = 0.45. We adopted the simpler TrN model in subsequent analysis because it is supported in most population genetic data analyses software packages.

Mitochondrial DNA diversity and phylogenetic structure

Screening of polymorphism within the 384-bp mtDNA control region fragment among 714 turtles from the 27 rookeries revealed 25 distinct haplotypes (Table 1 and GenBank accession numbers S76889 and AY955198–AY955221; haplotype nomenclature follows Norman *et al.* 1994 and is drawn from a global analysis of mtDNA variation in green turtles; D. Broderick, unpublished). Of 45 polymorphic sites, 41 were transitions, one site contained both a transition and a transversion and three sites were characterized by indels. A single base insertion distinguished the B5 haplotype and a single base deletion was unique to the A1 haplotype. The E1 and E2 haplotypes shared a 10 (bp direct duplication not seen in the other haplotypes. This same duplication was reported among phylogenetically independent haplotypes from Atlantic Ocean rookeries (Lahanas *et al.* 1994). The 25 haplotypes differed by between 1 and 35 observed mutations, corresponding to estimated sequence divergences of 0.3–10% (mean = 5.2%). Maximum-

likelihood and maximum-parsimony trees produced similar topologies with the latter tending to have higher levels of bootstrap support for each node (Fig. 2). There were five well-supported clades on the phylogenetic tree with 1 to 10 haplotypes each, separated by 3.3–8.4% mean sequence divergence (Fig. 2). Within clades, sequence divergence was low (0–1.3%). The 95% confidence parsimony networks could be constructed within, but not across the five clades because of large interclade divergences (Fig. 2).

Population diversity and subdivision

Despite the identification of five divergent clades, phylogeographical structure was diminished by the occurrence of several widespread haplotypes (Table 1, Fig. 2). For example, the numerically dominant (and in the network, centrally located) C3 haplotype of clade I was widely distributed across the Indian Ocean and Southeast Asia, and had limited occurrence in the Pacific Ocean. The rest of clade I (C1, etc.) and clade V (A1, etc.) were also widespread. Clade I was predominant in the Arafura Sea rookeries and across the Sunda Shelf to the east Indian Ocean. Clade V predominated in the Pacific Ocean, but also occurred in rookeries of the western Indian Ocean. Clade II (B1/3/4) occurred mostly, but not exclusively, in Pacific Ocean rookeries, as did clade III (C12/13–J1/2). Clade IV (B5) was only observed from the Sunda Shelf samples.

Exact tests for divergence of haplotype frequencies among all 27 rookeries revealed that 17 of the 351 pairwise comparisons were nonsignificant ($P > 0.05$). Of the nonsignificant comparisons, nine involved geographically proximal rookeries: Heron, Lady Musgrave and Northwest islands of the sGBR (= 96 km apart, $P = 0.34–0.68$); Raine Island/No. 8 Sandbank and Bramble Cay from the nGBR (274 km apart, $P = 0.56$); Bountiful Island, Groote Eylandt and Port Bradshaw from the Gulf of Carpentaria (= 567 km apart, $P = 0.17–0.56$); Paka and Redang islands from Peninsular Malaysia (133 km apart, $P = 0.14$); and the Malaysian and Philippine 'Turtle Islands' (14 km apart, $P = 0.76$). Two sets of comparisons showed no significant divergence in haplotype frequencies between more distantly separated rookeries. The first involved two rookeries from the Northwest Shelf of Australia, the Lacepede Islands and the Northwest Cape Jurabi coast (997 km apart, $P = 0.79$). The second involved the three Micronesian rookeries of the Elato, Ngulu and Ulithi atolls (= 1026 km apart, $P = 0.22–0.71$). Each of the remaining four nonsignificant ($0.18 < P < 0.75$) pairs of comparisons involved a single rookery from one of the above statistically homogeneous groupings and another distant rookery. We combined geographically proximal rookeries that did not have significantly different haplotype frequencies (i.e. Peninsular Malaysia, Sulu Sea, Micronesia, Northwest Shelf, GoC, nGBR, and sGBR). Using these groupings, the Exact tests were repeated, now among

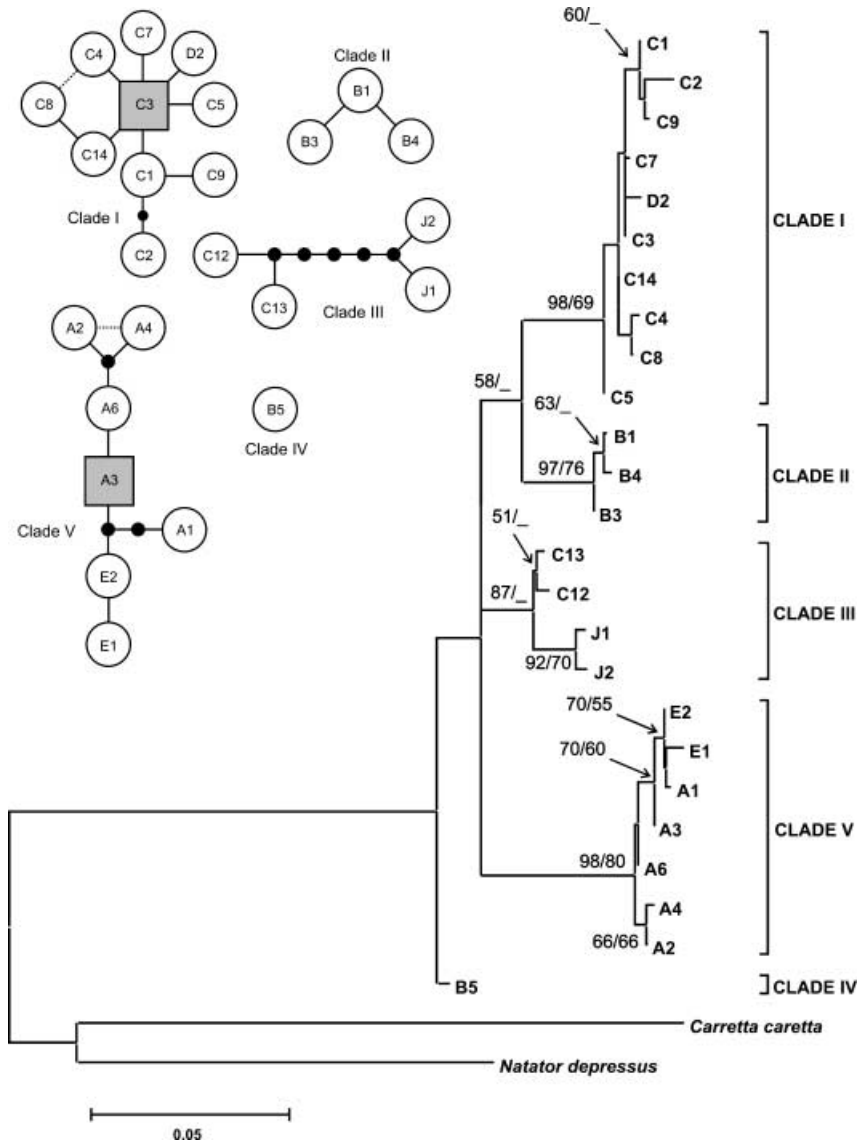


Fig. 2 Green turtle phylogeny and the estimated 95% set of plausible parsimony networks (top left) describing the relationships among 25 mtDNA control region haplotypes. The phylogeny shows percentage bootstrap support from a maximum parsimony (before slash) and a maximum-likelihood heuristic search (after slash) using TrN distance measures and 200 and 1000 bootstrap iterations, respectively. Branch lengths are proportional to the percentage sequence divergence indicated by the scale. Haplotypes within the haplotype network connected by solid lines are one mutational step away from each other; alternative parsimonious connections are represented by dotted lines. Presumed ancestral haplotypes are represented by shaded squares and unsampled intermediate haplotypes are represented by solid circles. Haplotype nomenclature follows Norman *et al.* (1994) and D. Broderick (unpublished) and is based on *Mse*I restriction digest patterns.

17 population groupings. Of the 136 pairwise comparisons, all were significant at a $P = 0.05$ threshold after correction for multiple tests using the sequential Bonferroni method (Rice 1989). Throughout the rest of the analyses these 17 groups were considered to represent MUs (Fig. 3).

Haplotype diversity varied widely among the 17 MUs ($h = 0.07-0.82$) and showed little consistent variation among regions (Table 2). Nucleotide diversity was substantially lower in the Southeast Asian MUs than the Indian Ocean MUs and these were both lower than the Pacific Ocean MUs ($\pi = 0.006, 0.019$ and 0.034 , respectively; Table 2). This difference reflects the dominance of closely related haplotypes from clade I in the Southeast Asian and, to some extent, the Indian Ocean populations, whereas the Pacific Ocean populations include haplotypes from multiple, distantly related clades (Table 1, Fig. 2).

Comparisons between estimates of long-term, female effective populations size (N_{ef}) derived from nucleotide diversity values and current estimates of the total number of nesting females (N_{af}) were equivocal. The ratio of N_{ef}/N_{af} within MUs ranged from 0.01 to 18.92 (Table 2). In the 16 comparisons that could be made, eight indicated that $N_{ef} > N_{af}$, six indicated that $N_{ef} < N_{af}$ and in two comparisons the estimate for N_{af} fell within the range estimated for N_{ef} . The average ratio of N_{ef}/N_{af} within MUs was 3.72 (median = 1.16) but there was a considerable range within each region. For example the Indian Ocean MUs had both the highest average ratio of N_{ef}/N_{af} (7.11) and the largest range (0.01–18.92) while the Pacific Ocean MUs had the lowest average ratio of N_{ef}/N_{af} (1.86) and the lowest range (0.19–5.32).

Of the 25 haplotypes we identified, 44% occurred uniquely in the Pacific Ocean, and 8% and 20% occurred uniquely in

Table 2 Estimates of haplotype (h) and nucleotide (π) diversity among 17 green turtle genetic stocks and estimates of effective populations sizes. N_{ef} is an estimate of the current population size. N_{ef} is the range of estimated effective population sizes based on mutation rates of $\mu = 0.009$ substitutions/site/million years

Region	Stocks (MUs)	n	h	SE	π	SE	N_{ef}	N_{af}	N_{ef}/N_{af}
Pacific	nGBR	52	0.35	0.08	0.019	0.010	4725	24300	0.19
	Coral Sea	41	0.43	0.08	0.030	0.015	7465	2800	2.67
	sGBR	102	0.15	0.04	0.010	0.006	2525	6600	0.38
	New Caledonia	10	0.82	0.10	0.042	0.023	10678	?	—
	Micronesia	49	0.68	0.06	0.004	0.003	987	1300	0.76
	PNG	18	0.22	0.12	0.017	0.009	4255	800	5.32
Pooled Pacific stocks		272	0.71	0.02	0.034	0.017			1.86
Southeast Asia	GOC	132	0.62	0.02	0.004	0.003	1026	6600	0.16
	Aru	28	0.07	0.07	0.006	0.004	1408	1000	1.41
	Berau Islands	29	0.78	0.03	0.008	0.005	2063	7100	0.29
	SE Sabah	30	0.58	0.08	0.002	0.002	447	300	1.49
	Sulu Sea	67	0.34	0.06	0.001	0.001	248	13900	0.02
	Sarawak	22	0.33	0.12	0.011	0.006	2842	300	9.47
Pooled Southeast Asian stocks		27	0.33	0.11	0.012	0.007	3118	350	8.91
Indian	Ashmore Reef	335	0.80	0.01	0.006	0.004			3.11
	Scott Reef	20	0.68	0.06	0.045	0.023	11349	600	18.92
	West Java	19	0.61	0.10	0.010	0.006	2576	300	8.59
	Northwest Shelf	23	0.40	0.09	0.001	0.001	273	300	0.91
Pooled Indian stocks		45	0.36	0.09	0.007	0.397	1645	125300	0.01
Overall Australasia		107	0.70	0.03	0.019	0.010			7.11
		714	0.88	0.01	0.041	0.020			

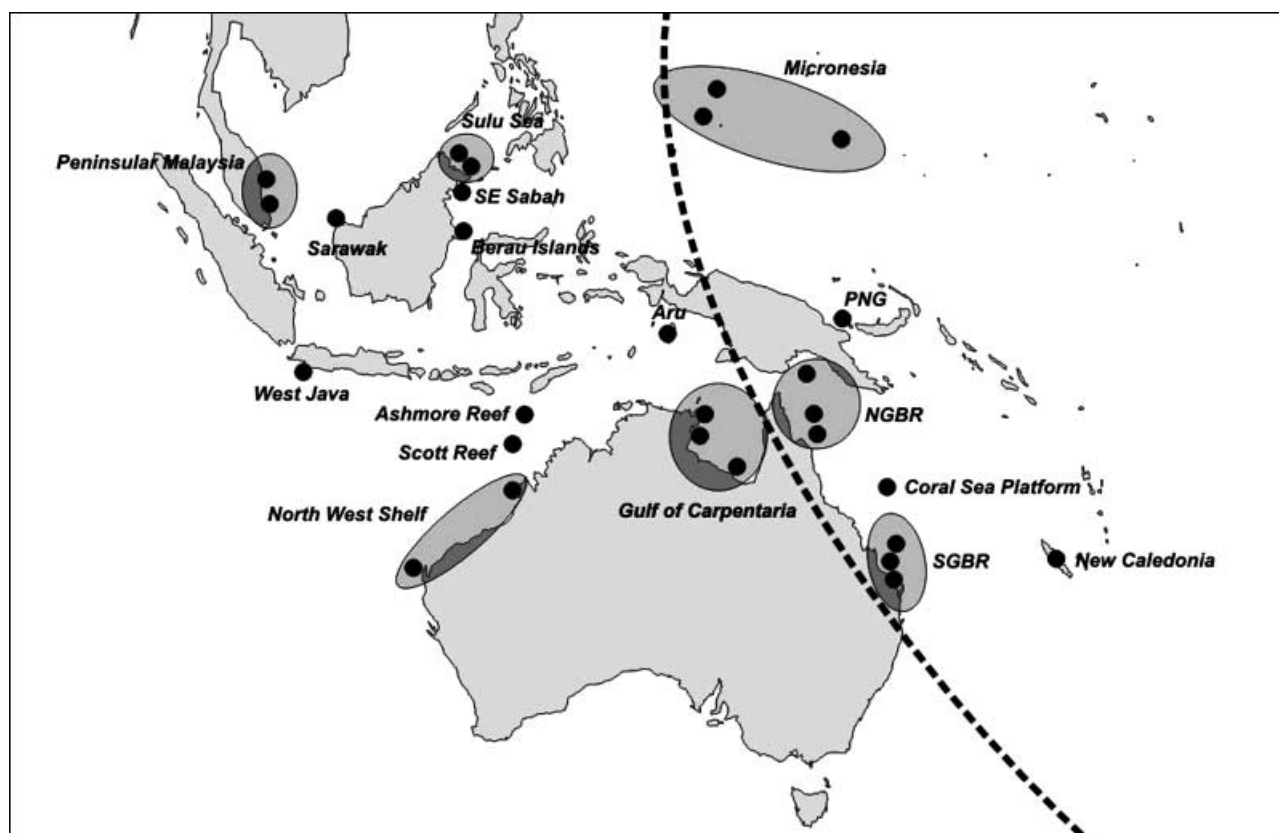


Fig. 3 Location of 17 genetically distinct breeding stocks or management units as inferred from analysis of geographical structure of mtDNA variants and position of the genetic barrier (dashed line), indicating the major genetic discontinuity between the Pacific Ocean rookeries from those to the west.

Table 3 Partitioning of molecular variance (%) of green turtle MUs at multiple geographical scales. All values are significant ($P < 0.001$)

	<i>n</i>	Φ_{ST} (TrN)			Conventional <i>F</i> -statistics		
		Among regions	Among stocks	Within stocks	Among regions	Among stocks	Within stocks
All stocks	17		77.6	22.4		52.8	47.2
Pacific stocks only	6		61.8	38.2		57.6	42.4
Indian stocks only	4		39.9	60.1		39.9	60.1
Southeast Asian stocks only	7		29.2	70.3		45.3	54.7
Stocks within regions		61.4	20.7	17.9	10.3	44.1	45.6

the Indian Ocean and Southeast Asian regions, respectively. The latter two regions were the least structured sharing 58% of haplotypes and the net sequence divergence between these regions was low (0.11%) compared to that between the Pacific and the Indian Ocean and Southeast Asian regions (4.03% and 5.16%, respectively). Analyses of molecular variance among MUs and among regions (Indian Ocean, Pacific Ocean, and Southeast Asia) indicated strong genetic structure ($P < 0.001$; Table 3). Overall, the proportion of variation distributed among the 17 MU was higher (78%) when molecular differences among haplotypes were included than when treating haplotypes as equidistant (53%), suggestive of some underlying separation of evolutionary lineages. The most striking effect was observed in a hierarchical analysis comparing regions; only 10.3% of genetic variation was partitioned among regions if considering only haplotype frequencies, vs. 61.4% if also considering haplotype divergence. However, this pattern was not consistent within regions. The incorporation of haplotype divergence into the AMOVA made no difference for the Indian Ocean comparisons, and it decreased the proportion of variance distributed among Southeast Asian MUs. Regardless of the approach, greater genetic variation within vs. among MUs was indicated for the Indian Ocean and Southeast Asia and the opposite was indicated for the Pacific Ocean. The Barrier analysis identified a major genetic discontinuity separating all Pacific Ocean rookeries from those to the west (Fig. 3). This phylogeographical break is also evident in AMOVAs; partitioning Pacific Ocean MUs from the other 11 MUs explains the greatest amount of genetic variation (68.4%) whereas only 1.54% of sequence variation was partitioned between Indian and Southeast Asian MUs.

Isolation by distance and migratory behaviour

A Mantel test for positive association between distance matrices of genetic structure ($F_{ST}/1 - F_{ST}$) and geographical distance was significant ($P = 0.001$) among the 27 rookeries sampled (Fig. 4a). However, the correlation (r^2) only explained 6% of the variance in these data. Analysis of spatial

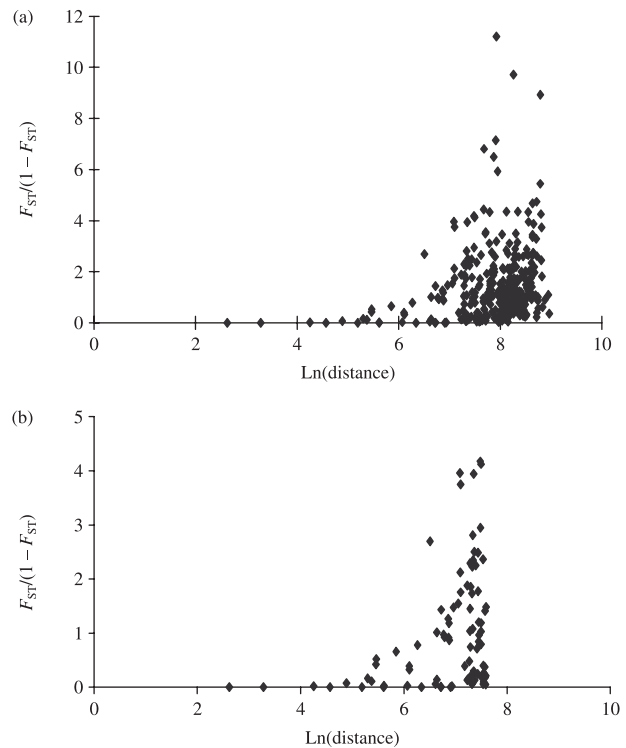


Fig. 4 Genetic differentiation among pairs of all Australasian green turtle populations (a) and populations separated by 2000 km or less (b), with regressions $y = 0.43x - 1.97$ and $r^2 = 0.06$; $y = 0.40x - 1.72$ and $r^2 = 0.12$, respectively. F_{ST} was linearized according to Slatkin (1993).

genetic autocorrelation indicated that genetic similarities between populations were significant when these populations were separated by up to 2000 km ($P = 0.011$; Fig. 5). At larger geographical distances there was no relationship between genetic and geographical distance. When correlation analyses were repeated only for rookeries separated by not more than 2000 km (using 1000 replicates), the correlation value (r^2) doubled to 12% (Mantel $P < 0.001$, Fig. 4b).

Along the east coast of Australia, the summer of 1998–1999 was one of the largest green turtle nesting events on

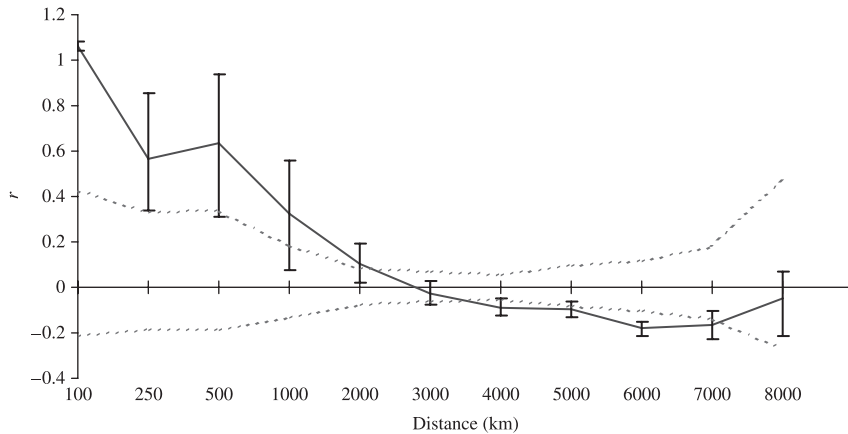


Fig. 5 Spatial autocorrelation analysis of green turtle stocks in the Indo-Pacific. The solid line represents genetic correlation as a function of geographical distance. Dotted lines indicate the 95% confidence interval about the null hypothesis of no spatial structure and were estimated from the 1000 permutations.

record and it provided an opportunity to compare the movements by nesting females among rookeries at multiple spatial scales. A total of 8156 tagged turtles were recorded during that season (Table 4), of which 2891 were remigrants from previous seasons. Of these interseasonal remigrants, 171 (6%) had changed rookeries between seasons and an additional 132 females (1.6% of the total) were observed to change rookeries between successive nesting events within the season. The subsequent mtDNA analysis showed that all of the migration events occurred within the identified MUs; in particular, the geographically extensive surveys of nesting females allowed for detection of nesting migrations between sGBR and nGBR, but none were observed. The rate of rookery switching for remigrants was somewhat higher within the sGBR ($151/1791 = 8.4\%$) than in the nGBR ($23/967 = 2.4\%$). Distances between alternative rookeries visited interseasonally by a single turtle ranged from 17 to 266 km (mean = 42 km) in the sGBR and from 12 to 218 km (mean = 65 km) in the nGBR. Within seasonal shifts between rookeries occurred over distances of 17–188 km (mean = 44 km) in the sGBR and 12–50 km (mean = 22 km) in the nGBR.

Discussion

Phylogeography and population structure

Green turtle populations sampled across Australasia had high levels of genetic diversity and showed evidence of a historical split between populations in the Pacific Ocean and those in the Indian Ocean and Southeast Asian region. However, the phylogeographical structure between the Pacific and Indian Oceans is less extreme than that between the Indo-Pacific and Atlantic Ocean (Bowen *et al.* 1992), as might be expected given the tropical marine connections between the Indian and Pacific Oceans.

Overall haplotype diversity of green turtles in Australasia was high ($h = 0.88$) and similar to Atlantic populations

($h = 0.83$, Encalada *et al.* 1996). Nucleotide diversities were elevated in Australasia ($\pi = 0.040$) compared to the Atlantic populations ($\pi = 0.005$) sampled over a similar geographical scale. However, within the Australasian region, the Southeast Asian rookeries had a pattern more like the Atlantic populations with high haplotype ($h = 0.80$) and low nucleotide diversities ($\pi = 0.006$). For the Atlantic green turtle populations, it was suggested that this pattern of variation reflects a relatively recent colonization by small founder groups from equatorial refugia, after sea temperatures increased at the end of the last glaciation (Encalada *et al.* 1996; see also Grant & Bowen 1998; Reece *et al.* 2005). In Australasia marine turtle rookeries would have been impacted substantially by glacial cycles; sea level changes have repeatedly exposed and flooded vast areas on the Sunda and Sahul shelves approximately every 100 000 years over the past 500 000 years (Chappell & Shackleton 1986; Torgersen *et al.* 1985). Most of the Southeast Asian green turtle nesting populations are in areas that were dry land at the last glacial maximum 18 000 years ago, when sea levels were 120–150 m below present (Chappell & Shackleton 1986; Voris 2000) and could only have been colonized between 10 000 and 6000 years ago as sea levels rose to their current levels. As new nesting habitat became available with rising sea levels, the most likely source of founders is nesting females from adjacent populations. The case of the Gulf of Carpentaria population on the Sahul Shelf is instructive about both the process of colonization and the potential for rapid divergence because the Gulf was flooded by marine waters from the west about 10 000 years ago (Torgersen *et al.* 1985). The distribution of mtDNA haplotypes suggests colonization of the Gulf of Carpentaria primarily from the west (now represented by the Northwest Shelf MU, which is known to use this region as a feeding ground, and the Timor Sea MUs) and perhaps the north (Aru MU). What is intriguing is that the GoC populations predominantly nest in the austral winter, whereas the Northwest Shelf, Timor Sea and

Table 4 Recapture data of green turtles (*Chelonia mydas*) nesting in eastern Australia during the 1998–1999 Australian summer nesting season, collected as part of an extensive combined State and Federal tagging programme. Values represent the number of turtles displaying between seasonal rookery fidelity (diagonal), between seasonal rookery switching (other) and within seasonal rookery switching (in parentheses). Saturation tagging of the total nesting population only occurred at Heron Island, Milman Island and Mon Repos. Other rookeries were sampled at mid season only

Rookeries where turtles were originally tagged			Rookeries examined in 1998–99 season																				
			MM	DS	PC	RI	N8	N7	CSC	SW	CI & FI	NW	WI	HI	HO	LM	LE	WR	RB	MP	MR	FR	
Northern Great Barrier Rookeries	Milman Is.	MM	96		1	3																	
	Douglas & Sinclair	DS	1 + (1)	—																			
	Moulter Cay	PC			1	7																	
	Raine Is.	RI			7 + (3)	847																	
	No. 8 Sandbank	N8				1	—																
	No. 7 Sandbank	N7				3	—																
	Coral Sea cays	CSC						133 + (2)															
Southern Great Barrier Rookeries	Swain Reefs cays	SW							3														
	Curtis and Facing Is.	CI & FI							3	1													
	Northwest Is.	NW								1	344		1 + (5)	10 + (9)	0 + (1)								
	Wreck Is.	WI									9 + (6)	382	8 + (12)	2			1					1	
	Heron Is.	HI									74 + (42)	16 + (34)	501	4 + (2)									
	Hoskyn Is.	HO												0									
	Lady Musgrave Is.	LM									2	4	4 + (3)	3	383	1	1						
	Lady Elliot Is.	LE													1 + (4)	0							
	Wreck Rock	WR									2				1		12					1 + (1)	
	Rules Beach	RB															0						
	Moore Park	MP																	1				
	Mon Repos	MR																		1			
	Fraser Is.	FR									1										1		
	Total tagged turtles		271	0	144	2069	0	0	1095	27	6	805	766	1801	76	740	99	65	4	4	3	15	181

Aru turtles predominantly nest in the austral summer. It appears that at some point, selective pressures have caused a shift in the timing of nesting within the last 10 000 years (c. 250 generations) as the Gulf of Carpentaria was colonized.

A strong feature of the mtDNA data for Australian green turtles is the distinction between the Pacific vs. Indian Ocean and Southeast Asian populations. The Pacific rookeries were dominated by haplotypes from clades I and IV, whereas the others had mostly haplotypes from clades II, III and V. This qualitative pattern was reinforced by the AMOVA and Barrier analyses. During the Pleistocene the intervening Torres Strait was repeatedly exposed, forming a land barrier between northeast Australia and New Guinea and it last reopened approximately 6000 years ago (Chappell & Shackleton 1986). Other genetic breaks due to this land barrier have also been observed in a variety of marine organisms including barramundi (*Lates calcarifer*; Chenoweth *et al.* 1998), coconut crab (*Birgus latro*; Lavery *et al.* 1996) and several other invertebrates (Benzie 1999). What is surprising is that despite recurrent opening of this barrier approximately every 100 000 years and the proximity of the large nGBR population, the genetic divergence has not been substantially eroded. Clearly natal philopatry at a subregional scale has provided an effective buffer against complete homogenization of these regions over long timescales.

Our estimates of historic effective size vs. current census population size produced highly variable results, but with many instances of $N_{ef}/N_{af} > 1$. This contrasts with the general pattern of effective population sizes being an order of magnitude smaller than census sizes (Frankham 1995), which might suggest major declines in current population size due to human impacts. Such estimates have been used previously to infer that populations are not in equilibrium due to historical fluctuations or recent reductions in population size (e.g. Lahanas *et al.* 1994). However, there are several caveats. First, the imprecision around point estimates for both N_{ef} and N_{af} is likely to be large because the former is based on a single gene and the latter is based on heterogeneous survey data. Second, admixture of divergent stocks during Holocene range expansions will inflate estimates of N_{ef} and even past admixture events can have a pronounced effect, as the time taken to return to equilibrium is proportional to N_e generations. Ideally, we would evaluate the demographic history of these populations and estimate current N_{ef} using coalescent simulations (e.g. Kuhner *et al.* 1998; Excoffier 2004; Hamilton *et al.* 2005). However, given the small number of generations (< 250), evidently low rate of substitution in mtDNA of marine turtles (Avisé *et al.* 1992), and the noise associated with parameter estimation from a single locus, we defer such analyses until a survey of microsatellite loci (N. FitzSimmons, in progress) is completed.

The geographical scale of contemporary dispersal

The combination of multiscale and intensive analyses of recaptures from physical tagging and genetic diversity has allowed for strong inference about the geographical scale of contemporary exchange among rookeries within and among regions. In eastern Australia the tag return data from the 1998–1999 nesting season showed an 8.3% dispersal among rookeries in the sGBR between nesting seasons for distances of around 250 km but no movements were observed between the nGBR and sGBR, separated by more than 1250 km. These single-season observations reflect those of long-term mark–recapture data from the major nesting sites in eastern Australia (Limpus *et al.* 1994, 2001, 2003). Crucially, these annual surveys include multiple nearby rookeries within regions (sGBR, up to 100 km; nGBR, up to 300 km apart) as well as between major breeding aggregations separated by larger distances (nGBR–sGBR, 1250 km). Data from a 28-year tagging programme on Raine Island and adjacent nesting sites in the nGBR showed that of 3662 females observed nesting across multiple years, 99% did so at the same rookery at which they were initially tagged (Limpus *et al.* 2003). Only 1% had shifted to other rookeries, mostly to nearby rookeries: Moulter Cay ($n = 16$; 14 km), no. 8 Sandbank ($n = 10$; 156 km) and no. 7 Sandbank ($n = 7$; 166 km). On one occasion a Raine Island turtle was recorded nesting on Bramble Cay (274 km). Similarly, 1.2% of turtles tagged while nesting on beaches within a 300-km radius from Raine Island were observed nesting on Raine Island in subsequent nesting seasons. Of particular interest, the mark–recapture surveys of the Raine Island rookery provided the only evidence that nesting movements among distant east Australian breeding sites can occur, albeit rarely. These records involved one female originally tagged while nesting on the Coral Sea Platform (850 km distant) and another originally tagged while nesting in the sGBR (at 1700 km); both later nested on Raine Island. These two events represent just 0.05% of the remigrants recorded. Conversely, no migrants from either the nGBR or the Coral Sea MUs have been observed nesting in the sGBR over the more than 30 years of intensive surveys. These cases also provide evidence that natal homing is not obligatory for green turtles, but rather that they do have the capacity to colonize new and distant habitat. The only other region where long-term mark–recapture data are available is at the Sulu Sea rookeries of the Philippines and Malaysian Turtle Islands. Here turtles shifted between rookeries separated by less than 100 km (DeSilva 1986; Trono 1993) within one nesting season.

Our genetic data are concordant with the mark–recapture data in demonstrating that gene flow among green turtle populations is likely to occur among proximate rookeries located within 500 km (e.g. within the nGBR, sGBR, GoC, Sulu Sea and Peninsular Malaysia MUs), but only rarely

among more distant ones. Given this behaviour we expected that *Chelonia mydas* would show a pattern of isolation by distance, wherein gene flow is effective at shorter geographical distances, but genetic drift prevails over longer distances (Hutchison & Templeton 1999). Indeed significant genetic correlation was apparent at smaller spatial scales (up to 2000 km) but, compared to other species with similar dispersal capacities, the overall association between genetic divergence and geographical separation was weak. Much stronger signatures were found in Indo-Pacific tasselfish (*Polynemus sheridani*), where 45% of the genetic differentiation ($P < 0.001$) was explained by geographical distances less than 3000 km (Chenoweth & Hughes 2003). Similarly, more than 50% of genetic variation in Pacific coral reef fish (*Acanthurus triostegus*) and 62% in the northwest Atlantic cod (*Gadus morhua*) were explained by distances up to 6200 km and 7000 km, respectively (Pogson *et al.* 2001; Planes & Fauvelot 2002). Pacific island populations of the coconut crab (*Birgus latro*) showed strong isolation by distance (60% of variation explained over 8000 km) and these were clearly different from the Indian Ocean populations (Lavery *et al.* 1996). The underlying assumption in tests for isolation by distance is that the populations under study are at migration–drift equilibrium (Wright 1943; Slatkin 1993) and, given the recent colonizations of the Sunda and Sahul shelves, we expect that the green turtle populations examined here violate this assumption. The repeated sea level changes during the late Pleistocene and associated exposure and flooding of beaches would have driven turtles to relocate and find new suitable nesting habitat, resulting in repeated and massive shifting of turtle populations across regions that at times had been isolated for thousands of years. Given the slow generation time in green turtles of 40 years (Limpus & Chaloupka 1997) and the inferred mutation rates of 0.006–0.012 (Encalada *et al.* 1996), the time elapsed since population colonization (6000 years) has not been sufficient for the populations to reach an equilibrium, which partially explains the weak association between geographical and genetic distance. Again, it would be more appropriate to use multilocus data and analytical methods that estimate contemporary or post-expansion migration rates (e.g. Pritchard & Donnelly 2001; Wilson & Rannala 2003; Hamilton *et al.* 2005).

Despite the limitations of the data for estimating population parameters, our analyses yield a useful qualitative result; the spatial scale of exchange observed in the intensive recapture study is consistent with that inferred from patterns of heterogeneity of mtDNA haplotype frequencies. Congruence between recapture results and mtDNA variation has been observed previously, but only at a broad spatial scale (Meylan *et al.* 1990). The multiscale recapture and mtDNA evidence from eastern Australia offers some validation to the widespread practice of delimiting demographically independent ‘management units’ of marine

turtles on the basis of distinctive mtDNA profiles (Moritz 1994; Avise 1995; Bowen 1995).

Implications for management

Many green turtle rookeries in Australasia have undergone precipitous declines over the last few decades, but others appear more stable (e.g. Limpus 1997; Chaloupka & Limpus 2001). Understanding the geographical scale at which rookeries are demographically connected vs. independent is central to diagnosis, management and monitoring of these populations (Moritz 1994; Bowen & Avise 1996). Our analyses of Australasian green turtles have identified 17 distinct MUs. All are present in single country jurisdictions, except for the Sulu Sea MU, which crosses the Malaysia/Philippine border. Where demographic units of nesting populations encompass multiple nations, they not only serve to focus management plans geographically but also to emphasize that conservation policies need to be regulated towards a common goal. The combination of genetic homogeneity and tag-return data across the Malaysian and Philippine Turtle Islands indicates regular interchange of females and thereby supports their joint management (Palma 1997). There is no such evidence for joint management units between Australia and neighbouring nations (Indonesia, New Caledonia and Papua New Guinea), given that all nesting beaches for each MU are contained within a single country. Whereas the tag-return data from eastern Australia show that individual turtles move between rookeries as far as 250 km apart, evidence of genetic exchange between several rookeries within 500 km suggests that movements at this scale are not uncommon, although rarely documented. As such a 500-km range typically provides a more accurate picture of the scale at which female movements occur and provides a guideline for conservation planning processes in this region. Additionally, turtles from each of these MUs cover a much greater geographical area during development and migrations between nesting and foraging locations; for example, Limpus *et al.* (2003) reports various instances where female turtles nesting in the nGBR were recaptured in foraging locations in the sGBR at > 1500 km distance or vice versa. Similarly, turtles were captured on feeding grounds in Aru, Indonesia that were originally tagged while nesting on Raine Island in the nGBR (K.D., personal observation), or the Lacepede Islands in Western Australia (J. P. Schulz, personal communication) and turtles nesting on sGBR beaches were recaptured at feeding grounds in Papua New Guinea, Solomon Islands and New Caledonia (Limpus 1992). As these migrations often cross international borders the importance of joint international management cannot be overemphasized. The larger countries (e.g. Malaysia, Indonesia and Australia) are typically supporting multiple MUs within their boundaries and their identification is

crucial to management by local communities, state and national agencies. For example, harvesting practices at one rookery can unknowingly have an impact on multiple populations at surrounding rookeries. Delineation of management areas for each management unit relies on a combination of tag returns, satellite tracking and genetic analysis of foraging and harvested populations all of which are currently being evaluated for this region.

Two features of green turtle populations in the Indo-Pacific are (i) that groups of adjacent rookeries that are isolated from other rookeries by more than a few hundred kilometres can be expected to support a genetically distinct management unit, and (ii) where a chain of adjacent rookeries extends over a large geographical area, the entire assemblage can be expected to represent a single management unit. Extrapolating from the findings above we can make predictions about the genetic affinities of some genetically unsampled but regionally significant rookeries. Rookeries from the northwest coast of West Papua and the coastal areas from Thailand through to Vietnam and China are likely to form two new management units based on their size and distance from to other management units. Rookeries off the West Kalimantan coast in the South China Sea are likely to be included within the Sarawak management unit and would benefit from a multinational management approach similar to that covering the Sulu Sea management unit.

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