

DNA BARCODING

**DNA barcodes for globally threatened marine turtles:
a registry approach to documenting biodiversity**EUGENIA NARO-MACIEL,*† BRENDA REID,‡ NANCY N. FITZSIMMONS,§ MINH LE,¶**
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Abstract

DNA barcoding is a global initiative that provides a standardized and efficient tool to catalogue and inventory biodiversity, with significant conservation applications. Despite progress across taxonomic realms, globally threatened marine turtles remain underrepresented in this effort. To obtain DNA barcodes of marine turtles, we sequenced a segment of the cytochrome *c* oxidase subunit I (COI) gene from all seven species in the Atlantic and Pacific Ocean basins (815 bp; $n = 80$). To further investigate intraspecific variation, we sequenced green turtles (*Chelonia mydas*) from nine additional Atlantic/Mediterranean nesting areas ($n = 164$) and from the Eastern Pacific ($n = 5$). We established character-based DNA barcodes for each species using unique combinations of character states at 76 nucleotide positions. We found that no haplotypes were shared among species and the mean of interspecific variation ranged from 1.68% to 13.0%, and the mean of intraspecific variability was relatively low (0–0.90%). The Eastern Pacific green turtle sequence was identical to an Australian haplotype, suggesting that this marker is not appropriate for identifying these phenotypically distinguishable populations. Analysis of COI revealed a north–south gradient in green turtles of Western Atlantic/Mediterranean nesting areas, supporting a hypothesis of recent dispersal from near equatorial glacial refugia. DNA barcoding of marine turtles is a powerful tool for species identification and wildlife forensics, which also provides complementary data for conservation genetic research.

Keywords: *Chelonia mydas*, COI, DNA barcoding, mtDNA, sea turtle, species identification

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Introduction

In recent years, DNA barcoding has become one of the leading international programmes to catalogue and inventory life on earth in light of current biodiversity loss (Hebert *et al.* 2004a, b; Hebert & Gregory 2005; Janzen *et al.* 2005; Savolainen *et al.* 2005; Smith *et al.* 2005). In this effort, data are collected from an agreed-upon DNA sequence in a standardized, rapid, cost-efficient and straightforward manner for species identification

purposes and to aid in species discovery (DeSalle *et al.* 2005; DeSalle 2006; Rach *et al.* 2008). Information from this unique identifier, the cytochrome *c* oxidase subunit I (COI, or *cox1*) gene, can offer the necessary resolution for distinguishing among species rapidly, providing insights into species diversification and molecular evolution (but see Moritz & Cicero 2004). DNA barcoding of threatened species provides an identification system for these species or their parts, allowing for rapid classification of illegally harvested organisms. The initiative enhances taxonomic understanding, which is key to developing appropriate conservation strategies (DeSalle & Amato 2004), and results can readily be made available to

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researchers, conservation practitioners, or other interested parties. Even so, prior to this study, globally threatened marine turtles were poorly represented in the DNA barcoding initiative.

Marine turtles have inhabited the Earth for over 100 Myr (Hirayama 1998), and occupy diverse ecosystems throughout their highly migratory life cycles (Bjorndal & Jackson 2003). After hatching from eggs on nesting beaches, the young disperse into the ocean. As juveniles, some species, including green (*Chelonia mydas*) and hawksbill (*Eretmochelys imbricata*) turtles, leave the pelagic environment and move to coastal feeding grounds, while others, including the leatherback (*Dermochelys coriacea*), continue to feed in the open ocean (Mussick & Limpus 1996; Hirth 1997). Adults undertake breeding migrations between feeding grounds and nesting areas that may be thousands of kilometres apart, and many females return to nest in the vicinity of their natal beach, a phenomenon known as natal homing (Carr 1967).

Marine turtles are threatened worldwide due to overharvest, fisheries interactions, habitat loss, climate change, pollution, disease and other factors, thus emphasizing the need for effective conservation measures, as well as the potential for DNA barcoding applications. There are seven widely recognized species of marine turtle (Table 1), as well as a distinct form of *Chelonia mydas* occurring in the Eastern Tropical Pacific whose taxonomic status has been debated (Kamezaki & Matsui 1995; Parham & Zug 1996; Pritchard 1996; Karl & Bowen 1999; Naro-Maciel & Le *et al.* 2008). All marine turtle species are listed under Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and included on the World Conservation Union's IUCN (2008) Red List of Threatened Species. Wildlife trade of these species can include meat, eggs, leather, shell and bone, for which the species or location of geographical origin may be difficult to identify using conventional means. In addition, animals caught as fisheries bycatch or stranding onshore may be damaged beyond recognition. By identifying these animals to species and providing a standardized registry for documenting genetic diversity within this group, DNA barcoding promises to advance conservation and research.

There are different ways to carry out species identification using DNA barcodes. In commonly used approaches, sequences are grouped using genetic distance, sometimes in combination with tree-building methods (Hebert *et al.* 2003a, b; Steinke *et al.* 2005; <http://www.barcodinglife.com>). Sequences can be assigned to the most similar group found in a BLAST search (Altschul *et al.* 1990). Genetic distances may also be used to build neighbour-joining trees (Tamura *et al.*

2004), and species assigned to the taxon they cluster with on these trees (Hebert *et al.* 2003a, b). However results may not be accurate if, for example, there is incomplete sampling in the database, and the nearest neighbour species is not the most closely related one (Koski & Golding 2001). Further, despite the wide usage of these methods, there is no threshold for genetic distance that can be used consistently to define species (Goldstein *et al.* 2000; Moritz & Cicero 2004; DeSalle *et al.* 2005). Overlap between inter and intraspecific divergence may present obstacles to correct assignment of query sequences, due to high intraspecific genetic variability or distances between species that are lower than within species (Meyer & Paulay 2005; Wiemers & Fiedler 2007; Rach *et al.* 2008). Consistent thresholds may also fail to be established due to variable effects of mutation rate and effective population size, among other factors. It is therefore useful to have a measure of certainty and risk in assignment of query sequences, and statistical methods are being developed to this end using a Bayesian framework (Nielsen & Matz 2006) and a decision theoretic and model-based approach (Abdo & Golding 2007; <http://info.mcmaster.ca/TheAssigner>).

These approaches also neglect to include information about diagnostic characters, or nucleotides that can be used to identify species and populations through their presence or absence, a method more consistent with classical taxonomy (DeSalle *et al.* 2005). Diagnostic characters, also referred to as characteristic attributes (CAs, Rach *et al.* 2008), can be classified as pure or private (DeSalle *et al.* 2005). Pure diagnostic characters are those shared among all elements in a clade, but absent from members of other clades at a node. Private diagnostic characters, on the other hand, occur in some members of a clade, but are not found in members of other clades at a node. CAs can be simple (occurring at a single nucleotide position; DeSalle *et al.* 2005) or compound (occurring at multiple nucleotide positions; DeSalle *et al.* 2005). By using CAs for diagnosis, error from incorrect grouping with the nearest neighbor is ruled out. By not relying on tree-building to assign species, the problem of using methods designed for hierarchically structured entities being applied to nonhierarchical groups, such as populations, is also avoided (DeSalle *et al.* 2005).

In this research, we provide the first barcode sequences for marine turtles of all extant species sampled in the Atlantic and Pacific, and investigate the utility of COI for barcoding purposes. We assess the marker's potential for species identification in marine turtles with relatively slow molecular evolution (Avice *et al.* 1992; FitzSimmons *et al.* 1995). We employ a character-based approach, the characteristic attribute organization system (CAOS; Sarkar *et al.* 2002a, b) and compare results to

Table 1 Sampling locations at nesting beaches in the Atlantic or Pacific Oceans for each of the seven sea turtle species, along with green turtles sampled from the Eastern Pacific and Mediterranean Sea. Also listed are number of haplotypes, their designations, GenBank Accession numbers (with the symbol ‘’ meaning ‘same as above’), number of pure diagnostic characters (Pu) and number of private diagnostic characters shared by at least 80% of group members (Pr)

Taxon	Atlantic			Pacific				
	Sample site (<i>n</i>)	# Haplotypes (Designation)	GenBank Accession numbers	Sample site (<i>n</i>)	# Haplotypes (Designation)	GenBank Accession numbers	Pu	Pr
<i>Caretta caretta</i>	Georgia, USA (5)	1 (CC-A1)	GQ152889	La Roche Percee, New Caledonia (2)	1 (CC-P1)	GQ152888	6	0
<i>Chelonia mydas</i>	Florida, USA (11)	1 (CM-A1)	GQ152881	Swain Reef, Australia (2)	1 (CC-P1)	''		
	Tortuguero, Costa Rica (8)	1 (CM-A1)	''	Dirk Hartog, Australia (2)	1 (CC-P1)	''		
	Quintana Roo, Mexico (3)	1 (CM-A1)	''	Cocos (Keeling) Islands, Australia (1)	1 (CM-P1)	GQ152877	11	1
	Cyprus (9)	1 (CM-A1)	''	Lacepedes, Australia (2)	1 (CM-P1)	''		
	Atol das Rocas, Brazil (35)	1 (CM-A2)	GQ152882	Port Bradshaw, Australia (1)	1 (CM-P1)	''		
	Trindade Island, Brazil (38)	1 (CM-A2)	''	Heron Island, Australia (1)	1 (CM-P2)	GQ152878		
	Suriname (10)	1 (CM-A2)	''	Scott Reef, Australia (1)	1 (CM-P2)	''		
	Ascension Island, UK (25)	1 (CM-A2)	''	Bramble Cay, Australia (2)	2 (CM-P3; CM-P4)	GQ152879; GQ152880		
	Guinea Bissau (2)	1 (CM-A2)	''					
	Aves Island, Venezuela (34)	2 (CM-A1, CM-A2, <i>n</i> = 32)	GQ152881; GQ152882					
<i>Chelonia mydas</i> of the Eastern Pacific								
	Mayumba, Gabon (7)	1 (DC-API)	GQ152876	Michoacan, Mexico (5)	1 (CM-P1)	GQ152877		
				Perth, Australia (2)	1 (DC-API)	GQ152876	30	0
<i>Dermochelys coriacea</i>				Solomon Islands (5)	1 (DC-API)	''		
<i>Eretmochelys imbricata</i>	Puerto Rico, USA (5)	1 (EI-A1)	GQ152887	Milman Island, Australia (4)	2 (EI-P1, <i>n</i> = 2; EI-P2, <i>n</i> = 2)	GQ152885 GQ152886	10	1
				Rosemary Island, Australia (4)	1 (EI-P2)	GQ152886		
<i>Lepidochelys kempii</i> *	New York, USA (5)	1 (LK-A1)	GQ152891				2	0
<i>Lepidochelys olivacea</i>	Ada Foah, Ghana (4)	1 (LO-API)	GQ152890	Oxley Islands, Australia (5)	1 (LO-API)	GQ152890	5	0
<i>Natator depressus</i>				Gulf of Carpentaria, Australia (2)	1 (ND-P1)	GQ152883	12	0
				Maret Island, Australia (1)	1 (ND-P1)	''		
				Barrow Island, Australia (2)	1 (ND-P1)	''		
				Duck Island, Australia (1)	1 (ND-P2)	GQ152884		
				Curtis Island, Australia (3)	1 (ND-P2)	''		

*Sample collected at a feeding rather than nesting area.

those obtained using typically employed phenetic and tree-building methods. We also discuss the applicability of a widely characterized genetic marker in marine turtles, the mitochondrial DNA (mtDNA) control region, for DNA barcoding purposes. We examine intraspecific variation over a wide geographical range to ensure comprehensive representation and seek evidence of cryptic species. We further explore the utility of the COI gene in shedding light on the group's evolutionary history and for population genetic applications. By obtaining DNA barcodes for globally threatened marine turtles, this study promises to aid in the enforcement of endangered species legislation, augment our knowledge of molecular evolution within this group and substantially contribute to the global DNA barcoding initiative's objective to document the diversity of life.

Materials and methods

Taxonomic sampling and laboratory analysis

We obtained blood or tissue samples from a wide global distribution for each species, and complemented this with a focused study of green turtles within the Atlantic Ocean and Mediterranean Sea. This resulted in 249 samples that were analysed from individual or multiple rookeries in the Atlantic and Pacific Oceans, the Mediterranean Sea and one feeding ground located in New York, USA (Table 1). DNA extractions were performed using a DNeasy Tissue Kit as per instructions for animal tissues or blood (QIAGEN Inc.) or by a salting out procedure. Polymerase chain reactions (PCR) were carried out using standard reagents and negative controls, with the primers L-turtCOI (5'-ACTCAGCCATCTTACCTGTGATT-3') and H-turtCOIc (5'-TGGTGGGCTCATACAATAAAGC-3') designed for a freshwater turtle by Stuart & Parham (2004). These primers were chosen because they span the COI segment utilized for DNA barcoding of other turtles (<http://www.barcodinglife.com>). PCR conditions were as follows: 95 °C for 5 min; 30–35 cycles of 95 °C for 45 s, 54 °C for 45 s, 72 °C for 45 s; 72 °C for 6 min followed by 4 °C storage. In rare instances where the sample was degraded, an additional PCR was performed using the PCR product as template. PCR products were then cleaned using the Ampure system with a Biomek automated apparatus. Sequencing reactions were conducted using standard protocols and BigDye reagents (PerkinElmer), followed by alcohol precipitations. PCR products were separated using an ABI 3730 sequencer, and sequencing was carried out in both directions. Alternatively, PCR products from Pacific region samples were cleaned using a polyethylene glycol protocol (Sambrook & Russell 2001) and sequenced by MacroGen. Sequences were aligned using the program Sequencher v4.6

(Gene Codes Corporation) and posted on GenBank and BOLD.

Data analysis

Genetic diversity. Mitochondrial haplotype (h) and nucleotide (π) diversities (Nei 1987) were calculated using the Arlequin program (v3.0; Excoffier *et al.* 2005). Variable sites, transition and transversion rates and coding differences in the whole data set were identified using MEGA v4 (Tamura *et al.* 2007). Haplotype networks based on statistical parsimony were constructed to elucidate relationships among COI haplotypes using TCS v1.21 (Clement *et al.* 2000).

Character-based diagnosis. We used the CAOS (Sarkar *et al.* 2002a, b) to identify diagnostic characters for species identification. We conservatively relied only on simple CAs, not including compound characters. We analysed pure CAs and private CAs with frequencies above 80%, following Rach *et al.* (2008). A guide tree was created using the maximum parsimony module in PHYLIP (v3.67; Felsenstein 2007) and incorporated into a NEXUS file containing COI sequence data in MacClade (v4.06; Maddison & Maddison 2002). Then, the P-Gnome program (Rach *et al.* 2008) searched each node, starting with the basal node, to identify diagnostic characters using the CAOS algorithm.

Genetic distance and tree-building. A BLAST search of GenBank was carried out using our COI sequences, and the species most closely matching our sequences was recorded. Intraspecific as well as mean interspecific pairwise distances were calculated using p -distances and the Kimura 2-parameter (K2P) distance model, commonly used in barcoding studies, in MEGA v4 (Tamura *et al.* 2007). MEGA was also used to create a neighbour-joining tree based on pairwise K2P distances for all COI sequences. Both of these analyses were performed through the online BOLD interface (Ratnasingham & Hebert 2007) as well, giving similar results.

Control region analysis. Character-based species diagnosis and analysis of genetic divergence were also carried out for publicly available mitochondrial control region sequences obtained for each marine turtle species from GenBank and the Archie Carr Center for Sea Turtle Research (<http://accstr.ufl.edu/genetics.html>). These sequences ($n = 367$ total) were aligned in MEGA and trimmed to a 395-bp common fragment to account for variations in sequence length. Of the publicly available sequences, 165 were from green turtles (*Chelonia mydas*, 65 from the Atlantic, 100 from the Pacific), 89 were from loggerhead turtles (*Caretta caretta*; 80 Atlantic, 9 Pacific),

19 were from leatherback turtles (*Dermochelys coriacea*; 9 Atlantic, 8 Pacific, and 2 described as occurring in the Atlantic and Pacific), 64 were from hawksbill turtles (58 Atlantic, 6 Pacific), 4 were from Kemp's ridley turtles (*Lepidochelys kempii*, Atlantic), 25 were from olive ridley turtles (*Lepidochelys olivacea*, 2 Atlantic, 23 Pacific) and 1 was from a flatback turtle (*Natator depressus*, Pacific). Any sequences that were from putative hybrids were excluded.

Results

Genetic diversity

Cytochrome *c* oxidase subunit I sequences were obtained from 249 individuals (815 bp; 271 amino acids). There were 159 variable sites in the data set, representing 19.5% of the data set, with T<->C transitions dominating. Most of the nucleotide changes were synonymous; however, two (0.7% of the data set) resulted in amino acid (AA) changes. These were AA 65: isoleucine to valine (*Dermochelys coriacea*) and AA 259: arginine to serine (*Eretmochelys imbricata*). The COI fragment was somewhat variable across marine turtle taxa, with haplotype and nucleotide diversities (Table 2) generally lower than or comparable to those reported for the mtDNA control region, although direct comparisons were not possible due to variation in sampling (Encalada *et al.* 1996, 1998; Bowen *et al.* 1998, 2007; Dutton *et al.* 1999; Shanker *et al.* 2004; Dethmers *et al.* 2006).

All COI haplotypes were separated into distinct networks by species using a 95% connection limit in *TCs* (Fig. 1). The number of haplotypes within hawksbill ($n = 3$) and green turtles ($n = 6$) was the greatest, while there were no COI sequence differences between ocean basins for olive ridley and leatherback turtles, with each represented by a single haplotype (Fig. 1; Table 2). Two different haplotypes were found in loggerhead turtles, each specific to an ocean basin. There were little or no differences among haplotypes within the species endemic to ocean basins: the Kemp's ridley, occurring only in the Atlantic, was characterized by a single haplotype,

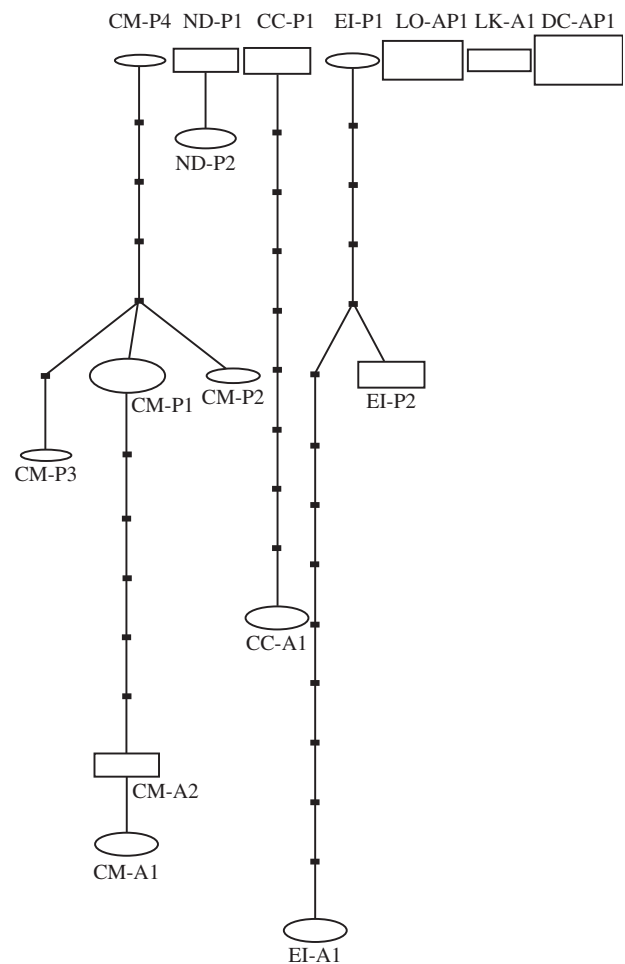


Fig. 1 COI haplotype network based on statistical parsimony. Haplotype designations correspond to those in Table 1. Lines indicate a single base pair substitution. The size of the circle or square is proportional to the haplotype frequency. Abbreviations are as follows: DC, *Dermochelys coriacea*; CM, *Chelonia mydas*; ND, *Natator depressus*; CC, *Caretta caretta*; EI, *Eretmochelys imbricata*; LO, *Lepidochelys olivacea*; LK, *Lepidochelys kempii*. Atlantic haplotypes are indicated by an A, Pacific haplotypes are indicated by a P, and those found in both ocean basins are indicated by an AP. The green turtle haplotypes were from Florida ($n = 5$) and Ascension Island ($n = 5$).

Species	Alleles	Haplotype diversity	Standard deviation	Nucleotide diversity	Standard deviation	Sample size
<i>Caretta caretta</i>	2	0.5455	±0.0722	0.00608	±0.00362	11
<i>Chelonia mydas</i>	6	0.3983	±0.0392	0.00143	±0.00103	188
<i>Dermochelys coriacea</i>	1	0.0000	±0.0000	0.00000	±0.00000	14
<i>Eretmochelys imbricata</i>	3	0.6667	±0.0782	0.00834	±0.00472	13
<i>Lepidochelys kempii</i>	1	0.0000	±0.0000	0.00000	±0.00000	5
<i>Lepidochelys olivacea</i>	1	0.0000	±0.0000	0.00000	±0.00000	9
<i>Natator depressus</i>	2	0.5556	±0.0902	0.00068	±0.00070	9

Table 2 Number of alleles, haplotype diversity (h) and nucleotide diversity (π), with sample size, of COI for marine turtle species

and the flatback, found only in the Pacific, displayed two similar haplotypes (0.07% divergence, Table 3; Fig. 1). No haplotypes were shared among species.

Character-based diagnosis

Character-based DNA barcodes were established for each a priori defined species using unique combinations of character states at 76 nucleotide positions (Table 4). Leatherback turtles were separated from all other marine turtle species by 30 diagnostic characters, while two CAs defined Kemp's ridleys. Diagnostic sites specific to ocean basins were found within green and hawksbill turtles. Atlantic hawksbill turtles were diagnosed by two T's at positions 430 and 753, while Pacific hawksbill turtles were diagnosed by an A at position 339, and a C at position 396. Atlantic green turtles were diagnosed by two T's at positions 240 and 573. However, no haplotypes diagnosed green turtle samples in the Eastern Pacific from other Pacific green turtles; indeed the haplotype from green turtles of the Eastern Pacific exactly matched that of green turtles sampled in Australia.

Genetic distance and tree-building

If COI sequences were assigned to the most similar group in a BLAST search of sequences posted on GenBank, the results would have only been partially correct. The

species with COI sequences already posted on GenBank were in fact most similar to their conspecifics. However, the remaining four species that did not have COI sequences posted on GenBank—leatherback, flatback, loggerhead and Kemp's ridley turtles—were most similar, in the BLAST search, to hawksbill, green, hawksbill and olive ridley turtles, respectively.

All mean values of intraspecific divergence at COI were below 1% (Table 3; Fig. 2), with pairwise K2P values of 0% for leatherback turtles and both ridley species, and ranging from 0% to 1.75% in hawksbill turtles, 0% to 0.12% in flatback turtles and 0% to 1.12% in loggerhead and green turtles. In Western Atlantic/Mediterranean green turtle populations, a gradient was detected for COI haplotypes. Turtles from most northern nesting sites (Florida; Costa Rica; Mexico; and Cyprus) were characterized by one haplotype, while those from southern or near equatorial nesting sites (Rocas and Trindade, Brazil; Ascension Island; Surinam) were fixed for a second haplotype (Fig. 3). A mixture of both haplotypes was found at Aves Island, Venezuela, a centrally located rookery, and the 'southern' haplotype was fixed in the eastern colony of Guinea Bissau (Fig. 3). Interspecific divergence levels using the K2P model ranged from 1.68% between the *Lepidochelys* species, to as high as 13.0% between green and leatherback turtles (Table 3; Fig. 2). Values produced using the BOLD program (Ratnasingham & Hebert 2007) were similar (data not shown). Trees based on COI

Table 3 Divergence values for: (A) COI (this study) and (B) D-loop (sequences from GenBank). Average within-species divergence calculated using the Kimura 2-parameter model (K2P) is on the diagonal. Average pairwise divergences between species are above (*p*-distance) and below (K2P) the diagonal

(A) COI divergence	<i>Caretta caretta</i>	<i>Chelonia mydas</i>	<i>Dermochelys coriacea</i>	<i>Eretmochelys imbricata</i>	<i>Lepidochelys kempii</i>	<i>Lepidochelys olivacea</i>	<i>Natator depressus</i>
<i>Caretta caretta</i>	0.63	8.56	10.65	7.59	5.04	5.55	8.20
<i>Chelonia mydas</i>	9.31	0.54	11.68	8.39	7.76	7.52	7.69
<i>Dermochelys coriacea</i>	11.70	13.01	0.00	9.43	10.15	10.53	10.72
<i>Eretmochelys imbricata</i>	8.15	9.07	10.02	0.90	7.02	7.15	9.14
<i>Lepidochelys kempii</i>	5.30	8.36	11.09	7.50	0.00	1.65	8.94
<i>Lepidochelys olivacea</i>	5.86	8.07	11.55	7.64	1.68	0.00	8.94
<i>Natator depressus</i>	8.84	8.31	11.86	9.94	9.73	9.71	0.07
(B) D-loop divergence	<i>Caretta caretta</i>	<i>Chelonia mydas</i>	<i>Dermochelys coriacea</i>	<i>Eretmochelys imbricata</i>	<i>Lepidochelys kempii</i>	<i>Lepidochelys olivacea</i>	<i>Natator depressus</i>
<i>Caretta caretta</i>	2.30	13.65	18.12	8.91	9.82	10.20	12.72
<i>Chelonia mydas</i>	15.30	4.96	20.80	14.35	14.06	13.96	11.80
<i>Dermochelys coriacea</i>	21.04	24.75	1.02	17.47	19.78	15.96	19.12
<i>Eretmochelys imbricata</i>	9.64	16.06	20.19	2.30	11.63	11.12	14.81
<i>Lepidochelys kempii</i>	10.69	15.70	23.31	12.73	0.00	6.01	13.00
<i>Lepidochelys olivacea</i>	11.13	15.62	18.09	12.15	6.35	1.48	14.40
<i>Natator depressus</i>	14.08	12.95	22.21	16.65	14.37	16.23	N/A

All values are given in percentages.

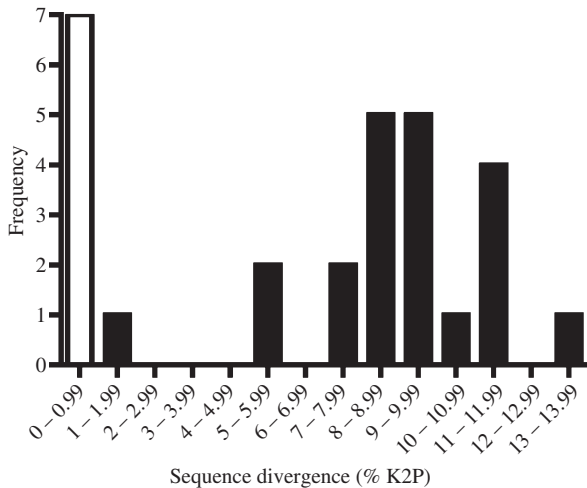


Fig. 2 Intra- and interspecific divergences in marine turtles calculated using the Kimura 2-parameter model. Intraspecific divergences are in white (mean = 0.27%; $n = 7$), and inter-specific divergences are in black (mean = 8.89%; $n = 21$).

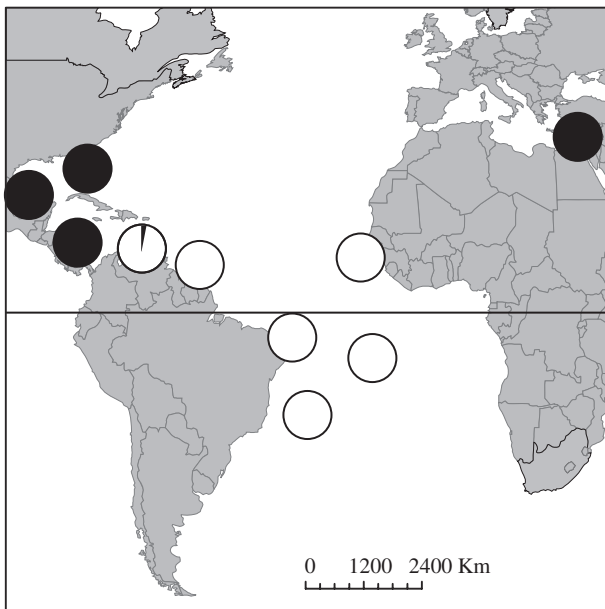


Fig. 3 COI haplotype frequencies of Atlantic and Mediterranean green sea turtle nesting areas, with respect to the Equator. Haplotype designations correspond to those in Table 1, with CM-A1 shaded black and CM-A2 shown in white.

sequences grouped species correctly with their conspecifics in all cases (data not shown).

Control region analysis

Character-based species diagnosis and tree-building using genetic distance were also carried out for mitochondrial control region sequences posted on

GenBank. No haplotypes were shared among species. However, at the more variable control region, no pure diagnostic characters were found for loggerhead, green, or olive ridley turtles, while private diagnostics at over 80% frequency were found for green turtles ($n = 7$). Of the remaining species, there were pure (Pu) and sometimes private (Pr) diagnostic characters defining leatherback ($nPu = 22$; $nPr = 1$), flatback ($nPu = 9$; $nPr = N/A$), hawksbill ($nPu = 8$; $nPr = 4$) and Kemp's ridley ($nPu = 2$; $nPr = 0$) turtles. Mean levels of genetic divergence were higher for the D-loop than for COI (D-loop divergence range using K2P model: interspecific: 6.35–24.75%; intraspecific: 0–4.96%; Table 3), and the range of pairwise divergences within variable species was larger (loggerhead turtles: 0–6.94%; green turtles: 0–12.28%; leatherback turtles: 0–1.69%; hawksbill turtles: 0–7.68%; olive ridley turtles: 0–4.61%; other species: N/A). In the neighbour-joining tree, all taxa grouped correctly with their conspecifics.

Discussion

DNA barcoding promises to be a powerful tool for species identification and other conservation genetic applications in marine turtles, which are unique on the evolutionary tree of turtles for occupying the marine realm, and widely known for their extensive migrations. Species identification, one of the main goals of the DNA barcoding initiative, was successfully carried out using their COI sequences. Even though these are ancient taxa with relatively slow molecular evolution (Avice *et al.* 1992; FitzSimmons *et al.* 1995), diagnostic sites were obtained for each of the seven marine turtle species at COI. Distance-based analysis of COI sequences consistently grouped members of the same species, although a complete baseline sample was necessary for correct assignment using phenetic methods. There was no convincing evidence of cryptic species revealed in this research, a result that is concordant with many other genetic studies of marine turtles. In addition, the barcodes provided insight into population structure and history. The COI marker was more suitable for barcoding objectives than mitochondrial control region sequences. However, hybridization is an important source of error for analyses relying solely on a mitochondrial marker, including in this group that is known to hybridize despite ancient separations (Conceição *et al.* 1990; Karl & Bowen 1995; Seminoff *et al.* 2003; Lara-Ruiz *et al.* 2006).

Cytochrome *c* oxidase subunit I barcodes were obtained for each of the a priori defined seven marine turtle species using unique combinations of their CAs (Table 4). The diagnostics were reliable, based on pure as well as private characters, with no haplotypes shared among species (Table 4; Fig. 1). On the highest end of the

range, 30 CAs diagnosed the leatherback turtle (Table 4). Of interest, five CAs diagnosed olive ridleys, while two diagnosed their sister taxon, Kemp's ridleys. There has been some debate about whether the ridleys are in fact separate species (Bowen *et al.* 1991), and the COI barcodes point to the validity of current species designations. For marine turtles, we found that the character-based approach was rapid through application of the CAOS algorithm using discrete characters, more consistent with classical taxonomy than distance-based methods and did not rely on somewhat arbitrary genetic distance thresholds for species identification. Importantly, the character-based approach was reliable—no species diagnoses could be made if the query sequences did not contain the relevant diagnostic characters.

On the other hand, query sequences could be assigned to the wrong species if a phenetic approach based on a BLAST search was employed in the absence of a complete baseline sample, such as the one available on GenBank prior to this study. For example, there were no leatherback COI sequences posted on GenBank, and a query on a leatherback sequence grouped it most closely with a hawksbill turtle. In the same vein, the remaining three species that did not have COI sequences posted on GenBank—the flatback, loggerhead and Kemp's ridley turtles—could be misidentified as green, hawksbill and olive ridley turtles, respectively; the species they were most similar to in COI BLAST searches.

Even so, these ancient marine turtle lineages did lend themselves well to distance- and tree-based barcoding approaches in some ways. There was no overlap between mean inter- and intraspecific distances, which many times introduces error into distance-based assignment of query barcode sequences (Meyer & Paulay 2005; Wiemers & Fiedler 2007; Rach *et al.* 2008). Most of the mean interspecific divergences were relatively high (range: 1.68–13.0%; Table 3), falling well above the typically used 2–3% threshold between inter- and intraspecific divergence (Hebert *et al.* 2003b; but see Moritz & Cicero 2004). The single exception was the lower level of divergence among the more recently speciated Kemp's and olive ridley turtles. Even so, due to low intraspecific variation within this genus, all of the turtles tested were accurately assigned to species using COI barcode trees. Mean intraspecific variation fell below 1% in all cases, fitting in well with the 2–3% threshold, and ranging from leatherback and olive ridley haplotypes that were identical across ocean basins, to more variable hawksbill turtle sequences (0–0.90%; Table 3).

Control region analysis

We considered the utility of mtDNA control region sequences for DNA barcoding purposes; given their

extensive use in sea turtle genetic studies (see Bowen & Karl 2007, for a review). The data are in many cases readily accessible: standardized mtDNA control region sequences are publicly available on GenBank and on other websites. Control region sequences have also been used for wildlife forensic purposes (Encalada *et al.* 1994).

We found that, although mtDNA control region sequences are of demonstrated utility for various conservation genetics objectives, they do not meet all DNA barcoding purposes as appropriately as COI sequences. At the more variable control region, pure or private diagnostic characters meeting a suggested reliability criterion of at least 80% frequency (Rach *et al.* 2008) were not found for several species. Even so, all species did group with their conspecifics in distance-based tree-building approaches. Inter- and intraspecific divergence levels were generally higher for the control region than for COI. In some cases, such as green turtles, mean intraspecific divergence levels close to 5% precluded establishing a 2–3% threshold demarcating inter- and intraspecific divergence. Also, one of the main benefits of COI barcoding is comparability to a wide range of taxa also being barcoded at this marker, which is not the case with the control region. Further, sampling was uneven as some species are vastly better represented than others on GenBank, an issue that may be considered in the context of developing statistical approaches, despite their computational intensiveness and/or inherent assumptions about the evolutionary process.

Cryptic species

The analysis provided no convincing evidence of new species units in most of the taxa examined. Leatherback and olive ridley turtle haplotypes were each identical across ocean basins, with no suggestion of hidden species units. These findings are consistent with previous work revealing shallow divergences between ocean basins in these species, likely due to recent colonization and population expansion (Bowen *et al.* 1998; Dutton *et al.* 1999). In fact, with the exception of Eastern Pacific green turtles (Kamezaki & Matsui 1995; Parham & Zug 1996; Karl & Bowen 1999) and the two species within the genus *Lepidochelys* (Bowen *et al.* 1991), there has been little recent debate over subspecific status in marine turtles. This study revealed that the COI sequence from green turtles of the Eastern Pacific was identical to a Pacific haplotype sampled in Australia, providing no evidence for species-level designation of Eastern Pacific green turtles based on this marker, and supporting conclusions of previous research (Bowen *et al.* 1993; Dutton *et al.* 1996; Karl & Bowen 1999; Naro-Maciel & Le *et al.* 2008). And, as noted above, each ridley species was characterized by

a single haplotype, and no haplotypes were shared among these taxa that are diagnosed by various CAs.

However, the study did uncover diagnostic characters specific to ocean basins within green and hawksbill turtles. These are both species in which there is a strong propensity for female natal homing, which differentiates populations at mitochondrial loci within ocean basins (Bass *et al.* 1996; Encalada *et al.* 1996; Dethmers *et al.* 2006; Formia *et al.* 2006; Velez-Zuazo *et al.* 2008). Deep divergence between Atlantic-Mediterranean and Indo-Pacific groups has been consistently reported in the literature for green turtles (Bowen *et al.* 1992; Encalada *et al.* 1996; Naro-Maciel & Le *et al.* 2008). Furthermore, these are tropical species whose dispersal across ocean basins tends to be limited by cold waters along the southern tips of continents. However, recent gene flow is known to have occurred between the Atlantic and Indian Oceans in green turtles (see Bourjea *et al.* 2006). We predict that increased sampling is likely to reveal other shared haplotypes between Atlantic and Indian Ocean populations, and that gene flow among these divergent lineages may be increased by changes to sea temperature, currents and sea levels, due to climate change. Thus although the COI diagnostics could serve as a flag for additional taxonomic investigation (Rach *et al.* 2008), the notion of cryptic species, or subspecies categories, does not appear warranted in marine turtles.

Population structure and history

Although COI analysis did not suggest to us that current species designations needed to be seriously challenged, it did indicate that barcoding could be useful for other conservation genetics purposes. For example, hawksbill, loggerhead and green turtles had haplotypes endemic to each ocean basin that could potentially be used to assign their origins. Additional sampling in the Indian Ocean and other areas would be of special interest in confirming the utility of COI to assign ocean basin origins in these groups.

Analysis of COI sequences revealed a north–south gradient in sequences from green turtles of Western Atlantic/Mediterranean nesting areas. Turtles from most northern nesting sites were characterized by one haplotype, while those from southern or near equatorial nesting sites were fixed for a second haplotype (Fig. 3). A mixture of both haplotypes was found at Aves Island, Venezuela, a centrally located rookery, and the ‘southern’ haplotype was fixed in the eastern colony of Guinea Bissau. These two haplotypes differed from each other by a single base pair (Fig. 3). These data are consistent with the hypothesis that turtles clustered in near equatorial regions during the most recent ice-age, and dispersed from these glacial refugia once the climate warmed about

10 000–18 000 years ago (Encalada *et al.* 1996). Rather than revealing an east–west clustering of rookeries (Encalada *et al.* 1996), however, the COI data suggest more of a north–south dispersal scenario.

In conclusion, the establishment of marine turtle COI barcodes may contribute to the global DNA barcoding effort to document and catalogue the diversity of life, particularly with regard to conservation applications. They have demonstrated utility for species identification and may additionally be useful for finer-scale assignment in some cases. Marine turtle DNA barcodes contribute to genomics science by increasing knowledge of COI across taxa. Through the Barcode of Life database (<http://www.barcodinglife.org/views/login.php>) and posting on GenBank, the results have been made readily available to researchers, conservation practitioners and other users. The barcodes can also be applied directly to the conservation of these globally endangered species when used to identify incidental sea turtle bycatch and illegally obtained or traded wildlife. Further, the barcodes enhance taxonomic understanding, which is central to developing appropriate conservation strategies (DeSalle & Amato 2004), and provide insight into population structure and history of this unique and highly threatened group.

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