NRC Research Press

ARTICLE

DNA barcode analysis of the endangered green turtle (*Chelonia mydas*) in Mexico[1]

Fátima Yedith Camacho-Sánchez, A. Alonso Aguirre, José Alberto Narváez-Zapata, Alan A. Zavala-Norzagaray, Cesar P. Ley-Quiñónez, H. Hugo Acosta-Sánchez, Hervey Rodriguez-González, Carlos Delgado-Trejo, and Miguel Angel Reyes-López

Abstract: Technological and analytical advances to study evolutionary biology, ecology, and conservation of green turtles (*Chelonia mydas*) are realized through molecular approaches including DNA barcoding. We characterized the usefulness of COI DNA barcodes in green turtles in Mexico to better understand genetic divergence and other genetic parameters of this species. We analyzed 63 sequences, including 25 from green turtle field specimens collected from the Gulf of Mexico and from the Mexican Pacific and 38 already present in the Barcode of Life Data Systems (BOLD). A total of 13 haplotypes were identified with four novel haplotypes from the Pacific Ocean and three novel haplotypes from the Atlantic Ocean. Intraspecific distance values among COI gene sequences by two different models were 0.01, demonstrating that there is not a subdivision for green turtle species. Otherwise, the interspecific distance interval ranged from 0.07 to 0.13, supporting a clear subdivision among all sea turtle species. Haplotype and total nucleotide diversity values of the COI gene reflect a medium genetic diversity average. Green turtles of the Mexican Pacific showed common haplotypes to some Australian and Chinese turtles, but different from the haplotypes of the Mexican Atlantic. COI analysis revealed new haplotypes and confirmed that DNA barcodes were useful for evaluation of the population diversity of green turtles in Mexico.

Key words: *Chelonia mydas*, COI gene, DNA barcodes, green turtle, Mexico.

Résumé : Des avancées technologiques et analytiques dans l'étude de la biologie évolutive, de l'écologie et de la conservation des tortues vertes (*Chelonia mydas*) ont été réalisées grâce à des approches moléculaires dont le codage à barres de l'ADN. Les auteurs ont examiné l'utilité des codes à barres COI chez les tortues vertes du Mexique pour mieux comprendre la divergence génétique et d'autres paramètres génétiques chez cette espèce. Les auteurs analysent 63 séquences dont 25 provenant de spécimens de tortues vertes échantillonnés dans le golfe du Mexique et la côte du Pacifique du Mexique et 38 séquences déjà présentes dans la base de

Received 1 December 2019. Accepted 28 January 2021.

F.Y. Camacho-Sánchez. Conservation Medicine Lab, Centro de Biotecnología Genómica-Instituto Politécnico Nacional, Reynosa, Tamaulipas, Mexico; Department of Environmental Science and Policy, George Mason University, Fairfax, Virginia, USA.
A.A. Aguirre. Department of Environmental Science and Policy, George Mason University, Fairfax, Virginia, USA.
J.A. Narváez-Zapata. Laboratorio de Biotecnología Industrial, Centro de Biotecnología Genómica-Instituto Politécnico Nacional, Reynosa, Tamaulipas, Mexico.
A.A. Zavala-Norzagaray and C.P. Ley-Quiñónez. Laboratorio de Vida Silvestre, Departamento de Medio Ambiente, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Unidad Sinaloa, Instituto Politécnico Nacional, Guasave, Sinaloa, Mexico.
H.H. Acosta-Sánchez. United Nations Development Programme-Comisión Nacional de Áreas Naturales Protegidas, Ciudad Victoria, Tamaulipas, Mexico.
H. Rodriguez-González. Laboratorio Nutrición Acuícola, Departamento de Acuacultura, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Unidad Sinaloa, Instituto Politécnico Nacional, Guasave, Sinaloa, Mexico.
C. Delgado-Trejo. Departamento de Ecología Marina, Proyecto Tortuga Negra, Instituto de Investigaciones sobre los Recursos Naturales, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Mexico.
M.A. Reyes-López. Conservation Medicine Lab, Centro de Biotecnología Genómica-Instituto Politécnico Nacional, Reynosa, Tamaulipas, Mexico.

Corresponding authors: Miguel Angel Reyes-López (emails: mareyesl@gmail.com; mreyesl@ipn.mx); A. Alonso Aguirre (email: aaguirr3@gmu.edu).

[1]This paper is part of a special issue entitled "Trends in DNA Barcoding and Metabarcoding 2020".

données BOLD. Au total, 13 haplotypes ont été identifiés dont quatre haplotypes inédits provenant de l'océan Pacifique et trois haplotypes inédits provenant de l'océan Atlantique. Les distances génétiques intraspécifiques parmi les séquences du gène COI, calculées au moyen de deux modèles différents, étaient de 0,01, ce qui montre qu'il n'y a pas de subdivision au sein des espèces de tortues vertes. Par ailleurs, l'intervalle de distances interspécifiques observées s'étendait de 0,07 à 0,13, ce qui confirme une subdivision claire entre les espèces marines de tortues vertes. La diversité des haplotypes et nucléotidique au sein du gène COI reflète une diversité génétique moyenne intermédiaire. Des haplotypes observés chez les tortues vertes de la côte du Pacifique du Mexique étaient partagés avec des tortues australiennes et chinoises, mais différents des haplotypes de la côte de l'Atlantique du Mexique. L'analyse du gène COI a révélé de nouveaux haplotypes et confirme que les codes à barres de l'ADN sont utiles pour évaluer la diversité des populations chez les tortues vertes du Mexique. [Traduit par la Rédaction]

Mots-clés : *Chelonia mydas*, gène COI, codes à barres de l'ADN, tortue verte, Mexique.

Introduction

As one of the most biodiverse countries in the world, Mexico provides extremely important nesting and foraging habitats for six of the seven recognized species of sea turtles, including leatherback (*Dermochelys coriacea*), green (*Chelonia mydas*), hawksbill (*Eretmochelys imbricata*), loggerhead (*Caretta caretta*), olive ridley (*Lepidochelys olivacea*), and Kemp's ridley (*Lepidochelys kempii*). The only species not found in Mexico is the endemic Australian flatback turtle (*Natator depressus*). All six species are classified as endangered of extinction by the Mexican Government and other civil international organizations. Efforts for their conservation and management have expanded for more than five decades (Pineda and Rocha 2016; SEMARNAT 2019; IUCN 2020).

The green turtle has a pantropical distribution and is known in the Mexican Caribbean as tortuga blanca (white turtle) and in the Mexican Pacific as tortuga prieta (black turtle) or Eastern Pacific green turtle owing to its different coloration stages (Parham and Zug 1996; Naro-Maciel et al. 2008; Boissin et al. 2019; Jensen et al. 2019). The black turtle is the most controversial taxonomically, as it is smaller and lighter, lays fewer eggs, and has a unique shape and color when compared to green turtles in other oceans (Okamoto and Kamezaki 2014; Álvarez-Varas et al. 2019). These characteristics have led to a classification by some as a separate species (*Chelonia agassizii*); however, most have classified it as a subspecies (*C. m. agassizii*) (Seminoff et al. 2013; Jensen et al. 2019).

Genetic studies have advanced our understanding of marine turtle biology and evolution; for example, the genetic diversity of sea turtles is determined by many biogeographic, behavioral, and ecological factors that provide specific characteristics of survival and adaptation to their environment (Frankham et al. 2002; Eizaguirre and Baltazar-Soares 2014). Their genetic structure and gene flux provide characteristics among populations for local adaptation and microevolutionary processes to maintain genetic diversity even in small populations (Pongratz et al. 2002; Reusche 2020). Marine turtles demonstrate natal philopatry in their selection of breeding sites and nesting beaches, resulting in diversity linked to regional or global genetic structure and nesting and feeding sites (Vargas et al. 2016). For example, their haplotype composition and nucleotide diversity are useful to link foraging aggregations to nesting sites in green turtles (Amorocho et al. 2012).

Research using DNA markers of green turtles was first reported with molecular studies on the evolutionary origin of an isolated Ascension Island nesting colony about 30 years ago (Bowen et al. 1989, 1992). Genetic surveys of green turtles around the world were expanded using restriction fragment length polymorphism (RFLP) markers (Avise and Bowen 1994). The use of mitochondrial microsatellite DNA techniques (Karl et al. 1992) has allowed an understanding of the gene flow related to mating and migration systems, providing the genetic tools for species conservation (Avise et al. 1992). Technological and analytical advances in the evolutionary biology, ecology, and conservation of green turtles have been possible by using several approaches including mitochondrial DNA (mtDNA) control region (CR or d-loop). These functional genetic markers used in divergence analyses have provided a better understanding of the ancestral demographic connections across ocean basins. In addition, quantitative PCR and transcriptomic and epigenetic markers have more recently been developed (Avise et al. 1992; Novelletto et al. 2016; Komoroske et al. 2017; Boissin et al. 2019; Jensen et al. 2019). Novel approaches such as environmental DNA and DNA metabarcoding have more recently been applied to provide a greater understanding of the ecological and physiological responses of sea turtles to their environment as related to anthropogenic change (Komoroske et al. 2017).

The Barcode of Life Data Systems, commonly known as BOLD or BOLDSystems (Ratnasingham and Hebert 2007), is a sequence database specifically devoted to DNA barcoding (Hebert and Gregory 2005). As of 2017, BOLD includes over 8.8 million DNA barcode sequences, from over 316 000 species (Hebert et al. 2004; Zahiri et al. 2017; Porter and Hajibabaei 2018). It also provides an online platform for analyzing DNA sequences making use of unique "codes" that are present in each cell of an organism in the form of species-specific fragments of the DNA nucleotide sequence (Reid et al. 2011).

BOLD contains few reported sea turtle sequences, including seven sequences for Kemp's ridley turtles and 38 sequences for green turtles (http://www.boldsystems.org/index.php/Public_SearchTerms).

Barcoding based on the gene sequences of cytochrome *c* oxidase subunit 1 mitochondrial gene (*cox*1 or COI) created a universal database of animal species, providing a much easier way to identify specimens, especially when using universal primers for the 5′-terminal region of the COI gene. COI gene sequences have also been used to construct haplotype networks, phylogenetic trees, nucleic divergence analyses, and for population genetic parameters (Hebert et al. 2003*a*; Morgada et al. 2015; Komoroske et al. 2017). COI is one of the most common markers to investigate the phylogeographic history within and among sea turtle species, improving our understanding of genetic diversity and phylogeography to support species management and conservation (Tikochinski 2020).

The objective of this study was to determine the usefulness of DNA barcoding of green turtles in Mexico using novel COI sequences and compare them with other COI sequences previously published in BOLD. In addition, a set of DNA variations or polymorphisms were determined for *C. mydas*. Furthermore, selected population genetic parameters were assessed to compare the COI gene and mitochondrial CR, to obtain additional information on this endangered species.

Materials and methods

Field specimens, COI sequences, and DNA Biobank

Field specimens were collected at four different sites in Mexico (Tables 1, 2) during the nesting process on arrival. Sterile blood collection tubes containing heparin (Vacutainer, Bristol Circle, Oakville, ON. USA) were used to collect blood from the dorsal occipital sinus (Owens and Ruiz 1980; Mettee 2014). Skin biopsies (1.5–2.5 mm^3 of tissue) were taken from the rear flipper using a 6 mm human-grade biopsy punch (Dermapunch, West Chester, PA, USA). Turtles were sampled (blood) in the Gulf of Mexico (GOM), specifically at Rancho Nuevo Sea Turtle Sanctuary, Tamaulipas (23.227526, −97.768468). Skin and blood samples were collected from both juvenile and adult green turtles in the Mexican Pacific, specifically in Bahía de Navachiste, Sinaloa (25.4658587, −108.8469954); Costa Azul, Angostura, Sinaloa (25.0785287, −108.1993456); and Isla Cleofas, Islas Tres Marias, Nayarit (21.3138754, −106.2683676). Subsequently, all turtles were released unharmed following previous protocols (Kutzari 2006).

Skin and blood specimens were stored at room temperature in 70% ethanol until total DNA was extracted as previously described (FitzSimmons et al. 1999). Also, 44 sequences were downloaded from BOLD, including 38 *C. mydas* sequences 5 sequences from other sea turtle species, and one African spurred tortoise (*Centrochelys sulcata*) sequence (TSNCM001-20), and used in this work (Tables 1, 2).

Amplification and sequencing of the COI gene segment

In the laboratory, genomic DNA was extracted using a Qiagen Dneasy® blood and tissue kit (QIAGEN, Valencia, CA), according to the manufacturer's protocol. Finally, all DNA samples were eluted in 50 μL (150 ng/μL) and stored at 4 °C until testing. Primers of the mitochondrial COI gene (Ward et al. 2005, 2009; Ivanova et al. 2007; Becker et al. 2011), Fish F1 (5′–TCAACCAACCACAAAG ACATTGGCAC–3′) and Fish R1 (5′–TAGACTTCTGGGTG GCCAAAGAATCA–3′), were used to amplify the COI gene in green turtle samples. The final mixture for the PCR reaction was 20 μL, which included 1 μL of DNA, 5 U of Taq polymerase (Bioline), 10 mM of dNTP's, 5× Tris-KCl as a buffer, 25 mM of $MgCl_2$, and 10 μM of each primer. The PCR reaction conditions were the following: 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 storage at 4 °C. Finally, the PCR products obtained were washed with an ethanol purification standard method, and then resuspended in sterile water. Subsequently, samples were unidirectionally sequenced in Eurofins Scientific (Eurofins Genomics LLC., Louisville, KY, USA). All reported sequences from BOLD were downloaded, aligned, analyzed, and compared to those sequences obtained in the field. A total of 10 random sequences were sequenced twice to ensure that sequences were accurate.

Data analysis

Two sets of 69 sequences were used in this work, including COI gene sequences originating from the 25 field samples collected in this study, specifically 13 in the Gulf of Mexico (GOM) and 12 in the Mexican Pacific, including seven skin biopsies and 18 blood samples, and 44 sequences downloaded from BOLD (Tables 1, 2), of these, 38 belonged to green turtles, five to other related sea turtle species, and one from a tortoise (TSNCM001-20). A total of 465 mitochondrial control region (CR) sequences (Tables S1, S2, S3[2]) were downloaded, of which 459 were retrieved from GenBank and the Archie Carr Center for Sea Turtle Research (ACCSTR, University of Florida, Gainesville, Florida, USA) and six sequences related to sea turtles came from GenBank, as controls.

All sequenced samples obtained in this work were checked by FASTQC software to perform quality control checks on raw sequence data. In general, FASTQC is used for high-throughput sequencing pipelines. FASTQC was not used for those sequences downloaded from public databases. Additionally, all sequences were transformed into the FASTA format type. Then, all were analyzed and aligned with Clustal W as implemented in the BioEdit v7.2.5 software (Larkin et al. 2007). Visual examinations and editing were performed for all inserted or deleted

[2]Supplementary data are available with the article at https://doi.org/10.1139/gen-2019-0213.

Table 1. Relationship of COI sequences for localities, codes, BOLD IDs, or GenBank accessions and number of sequences and specimens collected from green turtles (*Chelonia mydas*) in Mexico, 2018–2019, and other previously published sea turtle sequences.

Origin	Species	Locality	BOLD ID/Genbank accession	Specimen	Latitude	Longitude
Gulf of Mexico	*Chelonia mydas*	Rancho Nuevo, Tamaulipas	CMMX001-20 CMMX002-20 CMMX003-20 CMMX004-20 CMMX005-20 CMMX006-20 CMMX007-20 CMMX008-20 CMMX009-20 CMMX010-20 CMMX011-20 CMMX012-20 CMMX013-20	Blood	23.227526	–97.768468
Pacific Ocean	*Chelonia mydas*	Bahía de Navachiste, Sinaloa	CMMX014-20* CMMX015-20* CMMX016-20* CMMX017-20* CMMX018-20*	Skin biopsy	25.4658587	–108.8469954
		Costa Azul, Sinaloa	CMMX019-20* CMMX020-20*	Skin biopsy	25.0785287	–108.1993456
		Isla Cleofas, Nayarit	CMMX021-20* CMMX022-20* CMMX023-20* CMMX024-20* CMMX025-20*	Blood	21.3138754	–106.2683676
Outgroup	*Eretmochelys imbricata*	Puerto Rico	BENT049-08.COI-5P, GQ152887	—	—	—
	Caretta caretta	Georgia, United States	BENT066-08.COI-5P, GQ152889	—	—	—
	Dermochelys coriacea	Mayumba, Gabón	BENT043-08.COI-5P, GQ152876	—	—	—
	Lepidochelys olivacea	Ada Foah, Ghana	BENT082-08.COI-5P, GQ152890	—	—	—
	Lepidochelys kempii[*]	New York, United States	BENT061-08.COI-5P, GQ152891	—	—	—
	Natator depressus	Queensland, Australia	BENT085-08.COI-5P, GQ152883	—	—	—

*Sample collected at a feeding rather than a nesting area.

sequencing chromatograms identified with erroneous nucleotides. Bioinformatic programs (further described below as DnaSP or IQTree) were used when it was necessary to eliminate gaps or to correct "N" letters. The downloaded sequence data from BOLD and corresponding accession numbers were summarized (Tables 1, 2). The complete alignments for COI and CR regions are uploaded as Files S1 and S2[2].

For COI sequences, Network 10.1.0.0 was used to obtain the mitochondrial haplotype networks (Nei 1987). Then, p-distance and the Kimura 2-Parameter model, K2P (Kimura 1980), implemented in MEGAX 10.1.8 (Kumar et al. 2018) were used to calculate pairwise genetic divergence among all sequences (Vargas et al. 2009).

To carry out haplotype networks, we used a complete control sequence in the COI gene of about 1562 bp tagged as GBMTG044-16.COI-5P or NC_000886 from BOLD ID or GenBank accession (Kumazawa and Nishida 1999), respectively. This sequence was aligned among the others in MEGA and a common fragment of 631 bp was spliced to consider the variations in sequence length. Additionally, for COI and CR, the fixation index or F_{ST} (Hudson et al. 1992) was obtained from the same DnaSP software to measure the difference in the allele frequency between both populations separately for each marker. Pairwise F_{ST} values were also calculated for COI and CR data within and between samples in each study area from GOM and the Pacific Ocean. *P* values were obtained by

Table 2. List of haplotypes according to origin, species, locality, code, and other data from collected and downloaded data of green turtles (*Chelonia mydas*) in Mexico, 2018–2019, and other previously published sea turtle sequences.

Origin	Haplotype	ID	Previous haplotype	BOLD ID	GenBank Accession	Locality
Atlantic Ocean/ Gulf of Mexico	Cm-A1A	014-08	CM-A1*	BENT014-08.COI-5P	GQ152881	Florida, United States
		015-08		BENT015-08.COI-5P		Florida, United States
		016-08		BENT016-08.COI-5P		Florida, United States
		017-08		BENT017-08.COI-5P		Florida, United States
		018-08	CM-A2*	BENT018-08.COI-5P	GQ152882	Ascension Island, United Kindom
		019-08		BENT019-08.COI-5P		Ascension Island, United Kindom
		020-08		BENT020-08.COI-5P		Ascension Island, United Kindom
		021-08		BENT021-08.COI-5P		Ascension Island, United Kindom
		022-08		BENT022-08.COI-5P		Ascension Island, United Kindom
		023-08		BENT023-08.COI-5P		Ascension Island, United Kindom
		5509-1	Unknown	CYTC5509-12.COI-5P	JX454972	Karpaz, Cyprus
		5516-1		CYTC5516-12.COI-5P	JX454990	Tortuguero, Costa Rica
		11171-		GBGC11171-13.COI-5P	JQ034420	Suriname
		11172-		GBGC11172-13.COI-5P	JQ026233	Costa Rica
		044-1		GBMTG044-16.COI-5P	NC_000886	Unpublished
	Cm-A2G	0701-18 LMC**	N/A	CMMX001-20**	N/A	Rancho Nuevo, Tamaulipas, Mexico
		0702_18 LMC**		CMMX002-20**		Rancho Nuevo, Tamaulipas, Mexico
		0703_18 LMC**		CMMX003-20**		Rancho Nuevo, Tamaulipas, Mexico
		0704_18 LMC**		CMMX004-20**		Rancho Nuevo, Tamaulipas, Mexico
		0705_18 LMC**		CMMX005-20**		Rancho Nuevo, Tamaulipas, Mexico
		0706_18 LMC**		CMMX006-20**		Rancho Nuevo, Tamaulipas, Mexico
		0707_18 LMC**		CMMX007-20**		Rancho Nuevo, Tamaulipas, Mexico
		0709_18 LMC**		CMMX009-20**		Rancho Nuevo, Tamaulipas, Mexico
		0710_18 LMC**		CMMX010-20**		Rancho Nuevo, Tamaulipas, Mexico
		0712_18 LMC**		CMMX012-20**		Rancho Nuevo, Tamaulipas, Mexico
	Cm-A3G	0708_18 LMC**	N/A	CMMX008-20**	N/A	Rancho Nuevo, Tamaulipas, Mexico
		0711_18 LMC**		CMMX011-20**		Rancho Nuevo, Tamaulipas, Mexico
	Cm-A4G	Cm-A4 G	N/A	CMMX013-20**	N/A	Rancho Nuevo, Tamaulipas, Mexico
	Cm-A5A	2936	N/A	GBGCR2936-19.COI-5P	KU958179	Italy
Pacific Ocean	Cm-PB1	1001_18 LMC	N/A	CMMX021-20	N/A	Isla Maria Cleofas, Nayarit, Mexico
		1002_18 LMC		CMMX022-20		Isla Maria Cleofas, Nayarit, Mexico
		1003_18 LMC		CMMX023-20		Isla Maria Cleofas, Nayarit, Mexico
		1004_18 LMC		CMMX024-20		Isla Maria Cleofas, Nayarit, Mexico
		1005_18 LMC		CMMX025-20		Isla Maria Cleofas, Nayarit, Mexico
		024-08	CM-PI*	BENT024-08.COI-5P	GQ152877	Michoacan, Mexico
		025-08	CM-PI*	BENT025-08.COI-5P	GQ152877	Michocan, Mexico
		026-08		BENT026-08.COI-5P		Michocan, Mexico
		027-08		BENT027-08.COI-5P		Michoacan, Mexico
		028-08	CM-PI*	BENT028-08.COI-5P	Unknown	Heron Island, Queensland, Australia
		030-08		BENT030-08.COI-5P		Heron Island, Queensland, Australia
		031-08		BENT031-08.COI-5P		Heron Island, Queensland, Australia
		032-08		BENT032-08.COI-5P		Heron Island, Queensland, Australia
		094-08	CM-PI*	BENT094-08.COI-5P	—	Michoacan, Mexico
		5508-1	Unknown	CYTC5508-12.COI-5P	JX454971	French Frigate Shoals, Hawaii, USA
		5510-1		CYTC5510-12.COI-5P	JX454974	Archipielago de Revillagigedo, Mexico
		11772-		GBGC11772-13.COI-5P	JX454978	Costa Rica
	Cm-PB2	1006-18 LMC	N/A	CMMX014-20	N/A	Bahía de Navachiste, Sinaloa
		1007-18 LMC		CMMX015-20		Bahía de Navachiste, Sinaloa
		1008-18 LMC		CMMX016-20		Bahía de Navachiste, Sinaloa
		1009-18 LMC		CMMX017-20		Bahía de Navachiste, Sinaloa
		1010-18 LMC		CMMX018-20		Bahía de Navachiste, Sinaloa
		1011-18 LMC		CMMX019-20		Costa Azul, Sinaloa
		1012-18 LMC		CMMX020-20		Costa Azul, Sinaloa
		137-0	Unknown	GBGC5137-08.COI-5P	EU600157	South China Sea, China

Table 2 (*concluded*).

Origin	Haplotype	ID	Previous haplotype	BOLD ID	GenBank Accession	Locality
	Cm-PB3	029-08	CM-P2*	BENT029-08.COI-5P	GQ152878	Heron Island, Queensland, Australia
		033-08		BENT033-08.COI-5P	Unknow	Heron Island, Queensland, Australia
		5512-1	Unknown	CYTC5512-12.COI-5P	JX454976	Yap, Federated States of Micronesia
		11766-		GBGC11766-13.COI-5P	JX454985	Sipadan, Malaysia
	Cm-PB4	034-08	CM-P2*	BENT034-08.COI-5P	GQ152879	Heron Island, Queensland, Australia
	Cm-PB5	035-08	CM-P2*	BENT035-08.COI-5P	GQ152880	Heron Island, Queensland, Australia
	Cm-PB6	2859	Unknown	GBGCR2859-19.COI-5P	KF894757	India
	Cm-PB7	5136-0		GBGC5136-08.COI-5P	EU600158	South China Sea, China
	Cm-PB8	2860		GBGCR2860-19.COI-5P	KF894758	India

*Sample collected at a nesting rather than a feeding area.

Arlequin 3.11 (Excoffier 2007). The exact test was performed to determine population differences in Arlequin 3.11. Field specimen sequences in Mexico were deposited in BOLD corresponding to accession numbers CMMX001-20 to CMMX013-20 from GOM and CMMX014-20 to CMMX025-20 from the Pacific Ocean. All new sequences originated in Mexico are available in BOLD upon publication of this work.

Tree-building for COI and CR markers

A best-fit model of sequence evolution by ModelFinder (Kalyaanamoorthy et al. 2017) was determined under the Bayesian Information Criterion (BIC). The TIM2+F+R2 and TIM+F+G4 models were chosen for the COI gene and CR sequences, respectively. The maximum-likelihood (ML) trees were constructed with IQ-TREE (v1.6.12) (Nguyen et al. 2015) with 1000 ultrafast bootstrap (BS) replicates (Minh et al. 2013). FigTree v1.4.4 (Rambaut 2010) was used to visualize the phylogenetic trees (Fig. 1) for COI and MEGAX for CR sequences (Fig. S1[2]).

Population genetic parameters

COI and CR sequence polymorphisms were analyzed by DNA Sequence Polymorphism (DnaSP) software v 6.12.03 (Rozas et al. 2017). These allowed the estimation of several values of DNA sequence variation, such as the number of variable sites (S), G + C content, and haplotype/nucleotide diversity. For the COI gene, the analysis was divided into several categories. First, all green turtle sequences were analyzed and compared with each other. Second, all sequences were grouped by haplotypes, then those groups were analyzed among them. Third, haplotypes were grouped according to the location where the specimen was collected. Lastly, sequences were grouped by ocean basins. The CR sequences were split in three categories: all sequences together, those from the Pacific Ocean, and those from the Atlantic Ocean.

Ethics statement

Research permits were granted in México by Dirección General de Vida Silvestre/Secretaría de Medio Ambiente y Recursos Naturales -SEMARNAT (Mexican Wildlife Department of the Secretary of Environment and Natural Resources) as field permits for the sampling, collection, and processing of blood samples (SGPA/DGVS/04674/10 and SGPA/DGVS/003769/18). All sea turtles were released at the location of capture.

Results

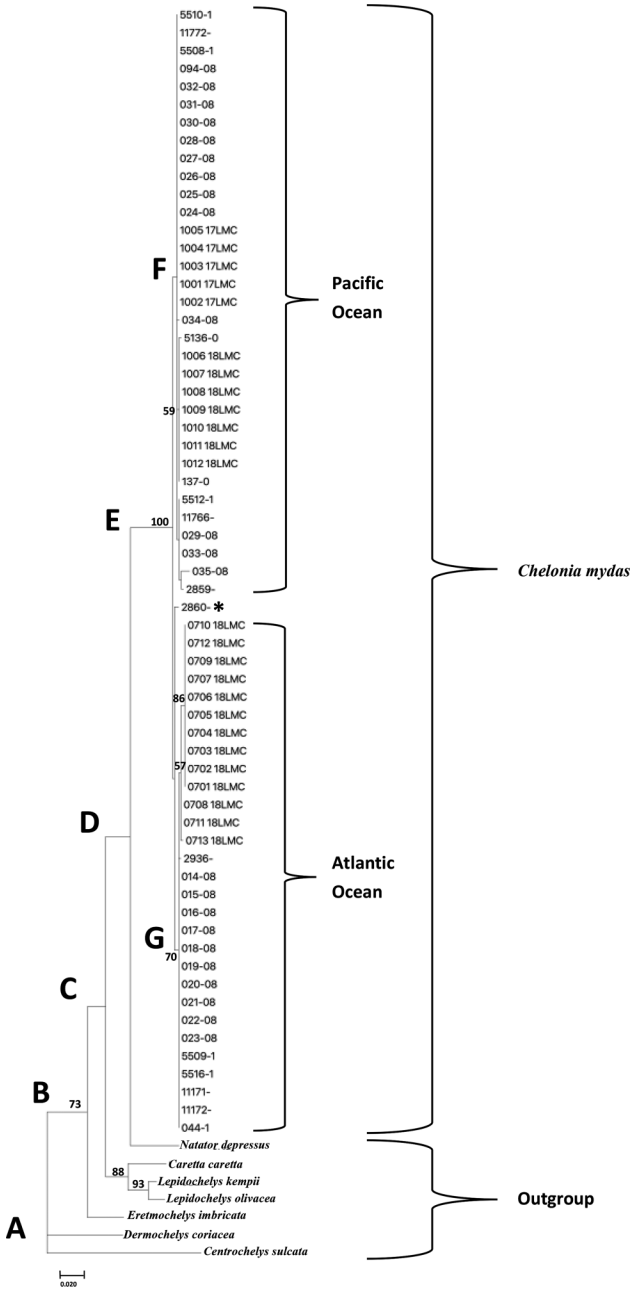
COI sequencing of all field samples yielded 632 bp fragments. The basic local alignment search tool (BLAST) was used to align the 25 COI sequences obtained in this work, against those sequences already reported in GenBank, obtaining a high identity range of 98%–100% in green turtles found in both oceanic basins. For all haplotypes (Tables 2, 3; Fig. 1), the 632 bp sequence of the COI gene was used in haplotype network analysis because it was the longest common length for all aligned sequences. For this alignment (Table 3), the first and last nucleotide (1 to 632) position of sequenced and downloaded data corresponded to positions 88 and 763, respectively, in the published consensus sequence of the COI gene.

A total of 13 haplotypes were identified in the network (Fig. 2), and this was confirmed using different analysis (Tables 2, 3). From these, five were identified as samples originating from GOM (Cm-A1A, Cm-A2G, Cm-A3G, Cm-A4G, and Cm-A5G) and eight from the Pacific Ocean (Cm-PB1 to Cm-PB8). No single haplotype was shared between these two groups of haplotypes.

Mitochondrial haplotypes analyzed using Network 10.1.0.0 for 63 COI sequences in green turtles showed a total of 16 bp with polymorphic nucleotides (2.5%) (Table 3). Besides, the intraspecific genetic distance value (Table 4) among individuals of green turtles was 0.01, using both, the K2P model and the *p*-distance model. Mean levels of genetic divergence for COI using the K2P model was 0.02–0.10 among green and sea turtles and 0.02–0.17 between all sea turtles and tortoise. The consensus sequence in green turtles had genetic distances between 0.07 and 0.13 and was within the range of 0.07–0.16 when they were compared with other sea turtles (7 different sea turtle species), and also between them and the tortoise species. On the other hand, when sea turtles were compared with the tortoise, genetic distances were more evident, ranging from 0.15 to 0.18.

Results from the phylogenetic study demonstrated the existence of five clades (Fig. 1). In general, these

Fig. 1. Maximum-likelihood tree depicting phylogenetic relationships for the 815 bp COI sequence in green turtles (*Chelonia mydas*), with related species sequences as an outgroup set. Clades are labeled with letters referenced in the text. Letter E indicates the green turtle main clade. The asterisk indicates a specimen from the Indian Ocean.



include the external groups, and a clear separation between genus and species of sea turtles and the land turtle was observed (letters A to D). Additionally, clades were obtained (letters D to E) that separate sea turtles by family and species. In clade (E) of green turtles, two separate subclades were identified, grouping the green turtle sequences, and the final clade of sequences split for the Pacific Ocean (F) and the Atlantic Ocean (G) including GOM were nested within a larger clade (E). Thus, all field samples collected for this study branched according to their geographic origin. Furthermore, samples from GOM were in a separate clade with BOLD sequences, classified as sea turtles from the Atlantic Ocean. In general, the same pattern was observed for the phylogenetic tree based on CR sequences in this species (Fig. S1[2]).

Also, a phylogenetic tree was built for CR sequences (Fig. S1[2]), which showed differences among all sea turtle species, including green turtles, and clearly shows two main clades for green turtles: one for the Atlantic Ocean and another for the Pacific Ocean, including the Indian Ocean sequences.

A comparison between the COI gene and CR was conducted to assess population gene parameters. For this analysis, the previously described sequences of the COI, 461 CR sequences (459 from *C. mydas* and 5 from other related species), were downloaded from BOLD and aligned and analyzed by DnaSP. The haplotype diversity (h) and nucleotide diversity (π) for the various markers are summarized in Table 5. The haplotype diversity for the COI population (h = 0.836), when all sequences were compared within both, the Pacific and Atlantic Oceans, was approximately 1/10 less than that estimated for the CR (h = 0.963). On the other hand, when populations were analyzed separately, the values decreased (h = 0.628, π = 0.002). However, when comparing only CR (h = 1.0) against COI haplotypes, the values were similar. For the CR, the nucleotide diversities (π = 0.074) are relatively high compared to that of the COI gene (π = 0.006). The haplotype and nucleotide diversity remain low compared to the value for CR, even when the analysis is divided by haplotypes, population, or ocean basin. In addition, values for CR according to ocean basins were determined. Similar results were obtained when these sequences were split from the total number of CR sequences (Table 5).

Pairwise F_{ST} values for the entire COI sample within and between sequences ranged from 0.29 to 0.70, depending on the type of analyses between populations and ocean basins (Table 6). Pairwise F_{ST} values for the entire sample (Table 6) of COI and CR sequences were 0.33 and 0.006, respectively.

Discussion

The COI barcode marker is used to perform high-throughput taxonomic assignments (Hebert et al. 2003*a*, 2003*b*; Porter and Hajibabaei 2018) and provide an important tool to support taxonomic studies of sea turtles. DNA barcoding improves the prospects for species-level identifications, using a standardized and authenticated DNA approach, and DNA barcodes are valuable in analyses of genetic divergences within and between species (Hebert et al. 2004; Vargas et al. 2009; Naro-Maciel et al. 2010). In this work, we successfully used blood and skin samples to obtain DNA and confirm what previous studies have demonstrated: that skin and blood specimens

Table 3. Mitochondrial haplotypes obtained for 63 COI sequences of green turtles (*Chelonia mydas*) from BOLD and field samples collected in Mexico, 2018–2019.

	POSITION																
HAPLOTYPE	16	81	102	177	339	375	402	489	490	510	585	624	627	630	631	632	Individuals
Cm-PB1	T	C	T	C	C	C	C	C	G	G	G	T	T	C	C	A	17
Cm-A1G				T		T		T		T	A						15
Cm-A2G				T		T		T		T	A	C			A	C	10
Cm-PB2												C					8
Cm-PB3					T												4
Cm-A3G				T		T		T		T	A	C					2
Cm-A4G	A			T		T		T		T	A	C					1
Cm-A5G				T		T		T		T	A			T			1
CM-PB8			C	T		T	T	T									1
Cm-PB4													C				1
Cm-PB5		A	C				T				A						1
Cm-PB6		A			T												1
Cm-PB7									A			C					1
	T	C	T	C T	C	C T	C	C T	G	G T	G A	T C	T	C	C A	A C	

Note: Nucleotide positions refer to the control sequence GBMTG044-16.COI-5P (BOLD ID), NC_000886 (GenBank accession). Cm-A and Cm-P are names for haplotypes used in this study. Colored letters represent the final consensus sequence in the COI gene and size of letters are frequency of each nucleotide in consensus sequences. Numbers below Position column represent the first and last nucleotide (88 to 763) position in control sequence that corresponded to positions 1 and 632 in sequenced samples performed during this research.

are suitable for DNA barcoding (Vargas et al. 2009). COI barcoding is ideal for identification of lost nests, turtles found stranded on the beach, or turtles caught by fishing (e.g., accidental by-catch). In addition, the application of this technique has clear forensic implications for illegal trade or poaching of eggs, meat, and other sea turtle products (Pilli et al. 2014; Yang 2018). Previous studies have demonstrated that sea turtles have high levels of heavy metals, and direct or accidental consumption represents a risk to human health (Aguirre et al. 2006). This is, to our knowledge, the first time that a comparison is made between green turtle sequences from Mexico by DNA barcoding with those previously reported in other parts of the world (Vargas et al. 2009; Naro-Maciel et al. 2010; Daza-Criado and Hernández-Fernández 2014).

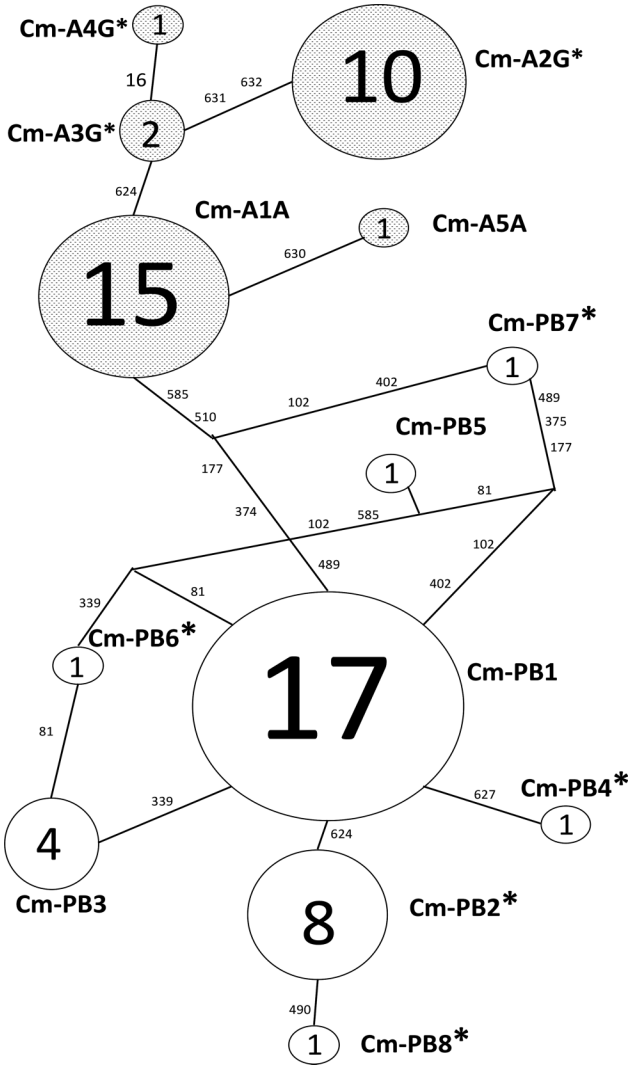
Other mitochondrial markers can also be used to infer the population history in turtles. One is the non-coding mitochondrial d-loop or control region (Barker et al. 2012) known to have a highly polymorphic nature. The nature of this segment should be considered before use, however, as technical complications have been reported, either at the time of performing the PCR, during sequencing, or both. Specifically, (*i*) the CR contains one or more regions of repeated expansion (which can be heteroplasmic), and (*ii*) CR exhibits segmental duplications. It should be clarified that not all the species studied present these problems (Barker et al. 2012). Nevertheless, many research groups prefer the use of coding genes, such as the COI gene.

Our study demonstrated five main clades of *C. mydas* using mtDNA (Fig. 1). Although the five clades are well supported, their branching pattern is not well resolved, particularly for the green turtle clade (E). In general, these results are similar to previous studies (Naro-Maciel et al. 2008, 2010; Vargas et al. 2009).

Mitochondrial COI gene haplotypes have been reported for all existing sea turtles (Vargas et al. 2009; Naro-Maciel et al. 2010; Daza-Criado and Hernández-Fernández 2014). However, our study provides a relevant contribution of new sequences (approximately 40%) from locations not reported before in Mexico nor in any part of the world. Previous investigations that have used the COI gene in DNA barcoding of sea turtles have documented 1–10 haplotypes (Vargas et al. 2009; Naro-Maciel et al. 2010). This work identified 13 haplotypes (Tables 2, 3, 4; Fig. 2). For the Atlantic Ocean/GOM, there were five haplotypes, of which three are novel. New sequences from GOM added to BOLD are not shared with those previously reported in Florida and Ascension Island, UK (Table 1), and they may serve as a reference for further population genetic studies.

There were eight haplotypes, Cm-PB1 to Cm-PB8, for the Pacific Ocean sequences; from these, four have never been reported (Cm-PB2, Cm-PB6, Cm-PB7, and Cm-PB8). Cm-PB5 and Cm-PB7 are placed in between the main branches from the Pacific and the Atlantic Oceans. Haplotype Cm-PB8 from the Indian Ocean, tagged 2860 in Fig. 1, clearly groups with the Pacific Ocean haplotypes in the haplotype diagram (Fig. 2). The last four haplotypes originated from different ocean basins. Based on previous studies, these matched an Australasian haplotype (Dethmers et al. 2006). Sequences obtained in the states of Nayarit and Sinaloa (Mexico) clearly grouped with the Pacific Ocean sequences; however, branches bifurcated geographically by state (Fig. 1). Sequences from Nayarit

Fig. 2. Haplotype network of green turtles (*Chelonia mydas*) using COI sequences obtained from BOLD and from specimens collected in Mexico, 2018–2019. Cm-A and shaded circles represent haplotypes from the Atlantic Ocean; Cm-PB and open circles represent haplotypes from the Pacific Ocean. Numbers in circles represent the number of sequences of each haplotype. Numbers on lines indicate positions where mutations were detected between two haplotypes. Asterisk indicates new haplotypes detected in this study.



belong to the main haplotype Cm-PB1 (Table 2; Fig. 2). This haplotype has been previously reported for Michoacan, Isla Maria Cleofas, Nayarit, and Revillagigedo Archipelago, Mexico; French Frigate Shoals; Hawaii, United States; and Queensland, Australia (Naro-Maciel et al. 2010), alluding to a possible migration corridor from Mexico to Australia or vice versa. On the other hand, haplotypes Cm-PB2 and Cm-PB3 correlated with previous sequences and haplotypes reported from the South China Sea (Tables 1, 2, 3). We hypothesize the presence of a similar potential corridor from Mexico to southern China for green turtles. A similar phylogenetic analysis recently published revealed the ancient origin of green turtles in the Indo-Pacific Ocean (Jensen et al. 2019).

A broader analysis with a larger number of sites and samples along those "corridors" will be required to identify common haplotypes. Our research verifies previous studies describing the split of green turtle sequences between the Atlantic and Pacific populations (Naro-Maciel et al. 2010; Jensen et al. 2019). Furthermore, sequences from specimens collected in GOM are new, and we speculate that this population is unique for this region (Fig. 1). Comparative results have demonstrated recent colonization of green turtles from the Central/Eastern Pacific and the Atlantic (Naro-Maciel et al. 2010; Boissin et al. 2019).

The values for intra- and interspecific distances in green turtles identified in this study are similar to those previously reported (Hebert et al. 2004; Daza-Criado and Hernández-Fernández 2014). Green turtle sequences, regardless of origin, had an extremely low variability, having a value of 0.01, as previously reported (Vargas et al. 2009; Naro-Maciel et al. 2010). DNA barcodes are valuable in the study of cryptic species (Trivedi et al. 2016), specifically for those morphologically similar but genetically different, such as the eastern Pacific green turtle (Zhang et al. 2019). Clustering analysis and haplotype networks suggest two distinct, major lineages for green turtles, one from the Atlantic and one from the Pacific Ocean (Naro-Maciel et al. 2010). However, we would need a stronger combination of genes and multiple markers from the nuclear genome to consider if these groups should be regarded species or subspecies (Komoroske et al. 2017; Jensen et al. 2019; Zhang et al. 2019).

The comparison of some population gene parameters (Table 5) using DnaSP software clearly demonstrated the potential of each marker. While COI is a conserved gene and its nucleotide diversity rate is low compared to CR, it can be used classically, as a gene for barcoding. This contrasts with the values presented by the CR of polymorphic sites (S), which are about 11 times higher than those observed for the COI gene. The results showed a high haplotype diversity (0.84 a 0.97) for both markers, but at the same time, they showed a low nucleotide diversity values, indicated by small differences between haplotypes. The values obtained for haplotype (h) and nucleotide diversity (π) in the COI analysis (Table 5) were comparable to those recently reported (Vásquez-Carrillo et al. 2020). The above data were consistently obtained by the two programs used, Arlequin and DnaSP. Probably, it is necessary to individualize and group by localities instead of grouping by ocean basins, given that various studies are carried out using this approach for green turtles (Naro-Maciel et al. 2010; Vásquez-Carrillo et al. 2020; Silver-Gorges 2020) or it could be that the analyses of the total of the sequences reflects a summative effect and that it is not possible to compare between populations. This has been indicated in works on green turtles from Colombia (Silver-Gorges 2020). In the case of COI

Table 4. Interspecific distance established for COI gene sequences within and among green turtles (*Chelonia mydas*) collected in Mexico, 2018–2019, and other previously published sea turtle sequences.

	Species	*Cm* BP	*Cm* AG	*Cm* AA	*Nd*	*Lo*	*Lk*	*Cc*	*Ei*	*Dc*	*Cs*
Pacific Ocean	*Chelonia mydas (Cm)* BP		0.01	0.01	0.07	0.07	0.08	0.09	0.08	0.12	0.14
Gulf of Mexico	*Chelonia mydas (Cm)* AG	0.01		0.00	0.07	0.08	0.08	0.09	0.08	0.12	0.14
Atlantic Ocean	*Chelonia mydas (Cm)* AA	0.01	0.00		0.07	0.08	0.09	0.08	0.12	0.12	0.14
	Natator depressus (Nd)	0.07	0.07	0.07		0.09	0.08	0.08	0.11	0.11	0.13
	Lepidochelys olivacea (Lo)	0.08	0.08	0.08	0.09		0.02	0.05	0.07	0.11	0.15
	Lepidochelys kempii (Lk)	0.08	0.09	0.09	0.09	0.02		0.05	0.07	0.10	0.15
Outgroup	*Caretta caretta (Cc)*	0.09	0.09	0.09	0.08	0.06	0.05		0.07	0.11	0.14
	Eretmochelys imbricata (Ei)	0.08	0.09	0.09	0.09	0.07	0.07	0.08		0.10	0.14
	Dermochelys coriacea (Dc)	0.13	0.13	0.13	0.12	0.12	0.11	0.12	0.10		0.15
	Centrochelys sulcate (Cs)	0.16	0.16	0.16	0.15	0.18	0.17	0.16	0.16	0.17	

Note: Mean pairwise divergences between species are below (K2P) the diagonal and above (*p*-distance). BP, AG, and AA are names for haplotypes used in this study.

Table 5. Population genetic parameters for green turtle (*Chelonia mydas*) mitochondrial clades in the COI gene and the control region.

Markers	Individuals	Nucleotide sites	Polymorphic sites (S)	G + C content	Haplotypes	Haplotype diversity (h)*	Nucleotide diversity (π)*
COI	63	632	16	0.436	13	0.836 ± 0.024	0.00633 ± 0.00032
Atlantic Ocean	29	632	5	0.433	5	0.628 ± 0.062	0.00251 ± 0.00024
Atlantic (Only)	12	632	1	0.432	2	0.167 ± 0.134	0.00026 ± 0.00021
Gulf of Mexico (Only)	17	632	4	0.433	4	0.618 ± 0.106	0.00242 ± 0.00037
Pacific Ocean sequences	34	632	11	0.440	8	0.697 ± 0.066	0.00214 ± 0.00053
Pacific Coast of Mexico (Only)	18	632	1	0.440	2	0.503 ± 0.064	0.00080 ± 0.0001
CR	459	287	172	0.327	170	0.962 ± 0.005	0. 05867 ± 0.00401
Atlantic	234	346	175	0.316	58	0.939 ± 0.009	0.01721 ± 0.00364
Pacific	187	289	161	0.318	107	0.990 ± 0.002	0.06368 ± 0.00860

*± SD.

Table 6. Pairwise F_{ST} values (below the diagonal) and *p* values (above the diagonal) of exact tests of population differentiation among green turtles (*Chelonia mydas*) based on 632 bp sequence mtDNA haplotypes.

GROUPS	FL	RN	ATL	CLEO_MICH	PAC	SIN_CA
FLORIDA (FL)		0.0	0.999	0.001	0.002	0.001
RANCHO NUEVO, MEX. (RN)	0.697		0.0	0.0	0.0	0.0
ATLANTIC OCEAN (ATL)	−0.128	0.707		0.0	0.0	0.0
CLEO_MICH, MEX*	1.000	0.779	0.913		0.004	0.0
PACIFIC OCEAN (PAC)	0.423	0.371	0.476	0.288		0.0
SIN_CA, MEX†	1.000	0.740	0.894	1.000	0.454	

*CLEO_MICH. Group formed by sequences in Cleofas Island and Michoacan.
†SIN_CA: Group formed by sequences in Sinaloa (Navachiste and Costa Azul), Mexico.

sequences from the Atlantic Ocean, the haplotype diversity value is 0.628; in other words, populations are not fixed. While the COI genetic divergence values can clearly differentiate between all the sea turtles, the values obtained for green turtles cannot discriminate between populations from the Atlantic and the Pacific oceans, as previously reported (Naro-Maciel et al. 2008, 2010; Vargas et al. 2009).

F_{ST} values can vary from 0 to 1, where 0 is equivalent to complete sharing of genetic material and 1 to no sharing (Long 2009). This was confirmed with values obtained at both basins (Table 6). F_{ST} for COI was larger (0.33) than CR (0.06). The genetic structure of both populations may be related to the new sequences obtained, specifically from Rancho Nuevo, Tamaulipas, which represents three new haplotypes (Fig. 2), adding to those previously reported (Naro-Maciel et al. 2010). While there are clear differences in ethologic features of this species, there are minimum molecular changes in the DNA barcodes obtained from different specimens.

The COI F_{ST} values for the Rancho Nuevo specimens ranged from 0.37 to 0.77 (Table 6), showing a larger

genetic difference within GOM than previously reported (Naro-Maciel et al. 2010). When comparing the Pacific and Atlantic populations, F_{ST} values ranged from 0.28 to 0.47. Further research is needed to assert these differences (Vásquez-Carrillo et al. 2020) and other markers should be used to strengthen the genetic analysis of green turtle populations, even if COI is useful for population studies (de Jong et al. 2011). For the Pacific, the F_{ST} value was high between SIN_CA and CLEO_MICH (1.00). However, F_{ST} values of 0.29 were reported in other Pacific Ocean populations (Silver-Gorges 2020), suggesting that they may be gene flow between them.

It is likely that the Rancho Nuevo population is isolated from GOM, and therefore, they do not reproduce with each other (Table 6). In the case of F_{ST} for the CR sequences, the values were similar to those found in other studies (Naro-Maciel et al. 2007; Vásquez-Carrillo et al. 2020). The CR brings together characteristics that have made it the marker par excellence in the study of sea turtles population structure because it is quick, simple, and informative as it has sufficient nucleotide variability. Nevertheless, it has limited resolution because it in reality only samples the female lineages (Komoroske et al. 2017). We show that, although also maternally inherited, COI adds information in the study of genetic diversity within *C. mydas*. An approach that combines other potential informative markers from the nuclear genome with data from CR and COI is recommended for future studies. A good place to start can be to use DNA markers that have been used to analyze genetic diversity in sea turtles (Roden and Dutton 2011; Hurtado et al. 2016; Xia et al. 2019).

This research has substantially expanded the sequence database for green turtles, both in number and in origin. Also, we report the first green turtle sequences from the Rancho Nuevo Sea Turtle Sanctuary in Tamaulipas, Mexico.

In conclusion, COI remains a good barcode marker for sea turtle species and contribute valuable information that can be used to differentiate and identify genera and species of this endangered group of organisms. Nevertheless, additional markers and samples from new sites should be added to perform a deeper comparison between populations of green turtles. The genetic and bioinformatic analysis of green turtles presented here may serve as a reference for future studies of this species across the world, documenting ecology and migratory movements, related to feeding, mating, or nesting sites, and allowing a more rational conservation effort for this endangered species.

Acknowledgements

This research was supported by SIP-20110067, 20161179, 20171851, 20196213, and 20200840 projects from the Instituto Politécnico Nacional (IPN). The Apoyos económicos de la Comisión de Operación y Fomento de Actividades Académicas (COFAA), Estímulos al Desempeño de los Investigadores (EDI), and Beca de estímulo institucional de formación de investigadores (BEIFI) from the IPN; the National System of Researchers (SNI); and the National Council of Science and Technology (CONACYT) provided fellowships and scholarships for M.A.R.-L., A.A.Z.-N., C.P.L.-Q., F.Y.C.-S., H.R.-G., and J.A.N.-Z. H.H.A.-S. is part of UNDP-GEF Project: species at risk and SEMARNAT/CONANP Tamaulipas. We are grateful to the fishing communities of Cerro Cabezón and Huitussi, Guasave, Sinaloa, especially the fishermen, A. Angulo Angulo, J.G. Angulo Machado, S. Báez, and R. Ayón Romo who supported part of the collection of sea turtle samples, Investigacion, Capacitacion y Soluciones Ambientales y Sociales A.C., Grupo tortuguero de las Californias A. C., Catherine E. Hart. We thank the entire community and Juan Martínez Ortíz of Rancho Nuevo, Aldama, Tamaulipas. Special thanks to Martha López Hernández, CONANP-Tamaulipas and Blanca Mónica Zapata Nájera, CONANP. Finally, the authors thank Claudia Patricia Larralde-Corona for grammar suggestions and Agusto Luzuriaga for his advice on bioinformatics. The publication fees were sponsored by the Department of Environmental Science and Policy, College of Science, George Mason University, Fairfax, Virginia.

References

Aguirre, A.A., Gardner, S., Marsh, J., Delgado, S., Limpus, C., and Wallace, N. 2006. Hazards associated with the consumption of sea turtle meat and eggs: a review for health care workers and the general public. EcoHealth, **3**: 141–153. doi:10.1007/s10393-006-0032-x.

Álvarez-Varas, R., Véliz, D., Vélez-Rubio, G.M., Fallabrino, A., Zárate, P., Heidemeyer, M., et al. 2019. Identifying genetic lineages through shape: an example in a cosmopolitan marine turtle species using geometric morphometrics. PLoS ONE, **14**: e0223587. doi:10.1371/journal.pone.0223587. PMID:31589640.

Amorocho, D.F., Abreu-Grobois, F.A., Dutton, P.H., and Reina, R.D. 2012. Multiple distant origins for green sea turtles aggregating off Gorgona Island in the Colombian Eastern Pacific. PLoS ONE, **7**: e31486. doi:10.1371/journal.pone.0031486. PMID:22319635.

Avise, J.C., and Bowen, B.W. 1994. Investigating sea turtle migration using DNA markers. Curr. Opin. Genet. Dev. **4**(6): 882–886. doi:10.1016/0959-437X(94)90074-4. PMID:7888759.

Avise, J.C., Bowen, B.W., Lamb, T., Meylan, A.B., and Bermingham, E. 1992. Mitochondrial DNA evolution at a turtle's pace: evidence for low genetic variability and reduced microevolutionary rate in the Testudines. Mol. Biol. Evol. **9**: 457–473. doi:10.1093/oxfordjournals.molbev.a040735. PMID:1584014.

Barker, F.K., Benesh, M.K., Vandergon, A.J., and Lanyon, S.M. 2012. Contrasting Evolutionary Dynamics and Information Content of the Avian Mitochondrial Control Region and ND2 Gene. PLoS One, **7**(10): e46403. doi:10.1371/journal.pone.0046403. PMID:23071562.

Becker, S., Hanner, R., and Steinke, D. 2011. Five years of FISH-BOL: Brief status report. Mitochondrial DNA. **22**: 3–9. doi:10.3109/19401736.2010.535528. PMID:21271850.

Boissin, E., Neglia, V., Boulet Colomb D'hauteserre, F.E., Tatarata, M., and Planes, S. 2019. Evolutionary history of green turtle populations, *Chelonia mydas*, from French Polynesia

highlights the putative existence of a glacial refugium. Mar. Biodivers. **49**: 2725–2733. doi:10.1007/s12526-019-01001-6.

Bowen, B.W., Meylan, A.B., and Avise, J.C. 1989. An odyssey of the green sea turtle: Ascension Island revisited. Proc. Natl. Acad. Sci. U.S.A. **86**: 573–576. doi:10.1073/pnas.86.2.573.

Bowen, B.W., Meylan, A.B., Perran, J.R., Limpus, C.J., Balazs, G.H., and Avise, J.C. 1992. Global population structure and natural history of the green turtle (*Chelonia mydas*) in terms of matriarchal phylogeny. Evolution, **46**: 865–881. doi:10.1111/j.1558-5646.1992.tb00605.x. PMID:28564410.

Daza-Criado, L., and Hernández-Fernández, J. 2014. Molecular identification and first report of mitochondrial COI gene haplotypes in the hawksbill turtle *Eretmochelys imbricata* (Testudines: Cheloniidae) in the Colombian Caribbean nesting colonies. Genet. Mol. Res. **13**: 7123–7132. doi:10.4238/2014.February.21.14. PMID:24634300.

de Jong, M.A., Wahlberg, N., van Eijk, M., Brakefield, P.M., and Zwaan, B.J. 2011. Mitochondrial DNA signature for range-wide populations of *Bicyclus anynana* suggests a rapid expansion from recent refugia. PLoS ONE, **6**(6): e21385. doi:10.1371/journal.pone.0021385. PMID:21731725.

Dethmers, K., Broderick, D., Moritz, C., FitzSimmons, N., Limpus, C., Lavery, S., et al. 2006. The genetic structure of Australasian green turtles (*Chelonia mydas*). Mol. Ecol. **15**: 3931–3946. doi:10.1111/j.1365-294X.2006.03070.x. PMID:17054494.

Eizaguirre, C., and Baltazar-Soares, M. 2014. Evolutionary conservation—evaluating the adaptive potential of species. Evol. Appl. **7**: 963–967. doi:10.1111/eva.12227.

Excoffier, L., Laval, G., and Schneider, S. 2007. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol. Bioinform. **1**: 47–50. PMID:19325852.

FitzSimmons, N.N., Moritz, C., and Bowen, B. 1999. Population identification. *In* Research and management techniques for the conservation of sea turtles. *Edited by* K. Eckert, K. Bjorndal, F. Abreu-Grobois, and M. Donelly. IUCN/SSC Marine Turtle Specialist Group, Publication No. 4.

Frankham, R., Ballou, J., Briscoe, D., and McInnes, K. 2002. Introduction to conservation genetics. Cambridge University Press. doi:10.1017/CBO9780511808999.

Hebert, P.D., and Gregory, T.R. 2005. The promise of DNA barcoding for taxonomy. Syst. Biol. **54**: 852–859. doi:10.1080/10635150500354886. PMID:16243770.

Hebert, P.D., Cywinska, A., Ball, S.L., and deWaard, J.R. 2003*a*. Biological identifications through DNA barcodes. Proc. R Soc. B Biol. Sci. **270**: 313–321. doi:10.1098/rspb.2002.2218. PMID:12614582.

Hebert, P.D., Ratnasingham, S., and deWaard, J.R. 2003*b*. Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proc. Biol. Sci. **270**: S96–S99. doi:10.1098/rsbl.2003.0025. PMID:12952648.

Hebert, P.D., Penton, E.H., Burns, J.M., Janzen, D.H., and Hallwachs, W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly *Astraptes fulgerator*. Proc. Natl. Acad. Sci. U.S.A. **101**: 14812–14817. doi:10.1073/pnas.0406166101. PMID:15465915.

Hudson, R.R., Slatkin, M., and Maddison, W. 1992. Estimation of gene flow from DNA sequence data. Genetics, **132**: 583–589. doi:10.1093/genetics/132.2.583. PMID:1427045.

Hurtado, L.A., De La Rosa-Reyna, X., Mateos, M., Shaver, D.J., Metz, R., Thiltges, J., et al. 2016. Thousands of SNPs in the critically endangered Kemp's Ridley sea turtle (*Lepidochelys kempii*) revealed by ddRAD-seq: opportunities for previously elusive conservation genetics research Gulf Mex. Sci. **33**: 214–218.

IUCN. 2020. Red list of threatened species. Available from http://www.iucnredlist.org [accessed 1 April 2020].

Ivanova, N.V., Zemlak, T.S., Hanner, R., and Hebert, P.D.N. 2007. Universal primer cocktails for fish DNA barcoding. Mol. Ecol. Notes, **7**: 544–548. doi:10.1111/j.1471-8286.2007.01748.x.

Jensen, M.P., FitzSimmons, N.N., Bourjea, J., Hamabata, T., Reece, J., and Dutton, P.H. 2019. The evolutionary history and global phylogeography of the green turtle (*Chelonia mydas*). J. Biogeogr. **46**: 860–870. doi:10.1111/jbi.13483.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., and Jermiin, L.S. 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods, **14**(6): 587–589. doi:10.1038/nmeth.4285. PMID:28481363.

Karl, S.A., Bowen, B.W., and Avise, J.C. 1992. Global population genetic structure and male-mediated gene flow in the green turtle (*Chelonia mydas*)—RFLP analyses of anonymous nuclear loci. Genetics, **131**: 163–173. doi:10.1093/genetics/131.1.163. PMID:1350555.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16**: 111–120. doi:10.1007/BF01731581. PMID:7463489.

Komoroske, L.M., Jensen, M.P., Stewart, K.R., Shamblin, B.M., and Dutton, P.H. 2017. Advances in the application of genetics in marine turtle biology and conservation. Front. Mar. Sci. **4**: 156. doi:10.3389/fmars.2017.00156.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol. Biol. Evol. **35**(6): 1547–1549. doi:10.1093/molbev/msy096. PMID:29722887.

Kumazawa, Y., and Nishida, M. 1999. Complete mitochondrial DNA sequences of the green turtle and blue-tailed mole skink: statistical evidence for archosaurian affinity of turtles. Mol. Biol. Evol. **16**(6): 784–792. doi:10.1093/oxfordjournals.molbev.a026163. PMID:10368956.

Kutzari, A.C. 2006. Manual de técnicas de protección de tortugas marinas. Kutzari/IFAW/WWF. 12 pp.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., et al. 2007. Clustal W and Clustal X version 2.0. Bioinformatics, **23**: 2947–2948. doi:10.1093/bioinformatics/btm404. PMID:17846036.

Long, J.C. 2009. Update to Long and Kittles's "Human genetic diversity and the nonexistence of biological races" (2003): Fixation on an index. Human Biol. **81**(5): 799–803. Available from https://www.muse.jhu.edu/article/381884.

Mettee, N. 2014. Sample collection techniques. Marine turtle trauma response procedures: a veterinary guide. WIDECAST Technical Report No. 17 [accessed online 13 February 2019].

Minh, B.Q., Nguyen, M.A., and von Haeseler, A. 2013. Ultrafast approximation for phylogenetic bootstrap. Mol. Biol. Evol. **30**(5): 1188–1195. doi:10.1093/molbev/mst024. PMID:23418397.

Morgada, M.N., Abriata, L.A., Cefaro, C., Gajda, K., Banci, L., and Vila, A.J. 2015. Loop recognition and copper-mediated disulfide reduction underpin metal site assembly of CuA in human cytochrome oxidase. Proc. Natl. Acad. Sci. U.S.A. **112**: 11771–11776. doi:10.1073/pnas.1505056112. PMID:26351686.

Naro-Maciel, E., Becker, J.H., Lima, E.H., Marcovaldi, M.A., and DeSalle, R. 2007. Testing dispersal hypotheses in foraging green sea turtles (*Chelonia mydas*) of Brazil. J. Hered. **98**: 29–39. doi:10.1093/jhered/esl050. PMID:17158465.

Naro-Maciel, E., Le, M., FitzSimmons, N., and Amato, G. 2008. Evolutionary relationships of marine turtles: A molecular phylogeny based on nuclear and mitochondrial genes. Mol. Phylogenet. Evol. **49**: 659–662. doi:10.1016/j.ympev.2008.08.004. PMID:18760371.

Naro-Maciel, E., Reid, B., Fitzsimmons, N.N., Le, M., Desalle, R., and Amato, G. 2010. DNA barcodes for globally threatened marine turtles: a registry approach to documenting biodiversity. Mol. Ecol. Resour. **10**: 252–263. doi:10.1111/j.1755-0998.2009.02747.x. PMID:21565020.

Nei, M. 1987. Molecular evolutionary genetics. Colombia University Press, New York. 512 pp. doi:10.7312/nei-92038.

Nguyen, L.T., Schmidt, H.A., von Haeseler, A., and Minh, B.Q. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. **32**(1): 268–274. doi:10.1093/molbev/msu300. PMID:25371430.

Novelletto, A., Testa, L., Iacovelli, F., Blasi, P., Garofalo, L., Mingozzi, T., and Falconi, M. 2016. Polymorphism in mitochondrial coding regions of Mediterranean loggerhead turtles: evolutionary relevance and structural effects. Physiol. Biochem. Zool. **89**: 473–486. doi:10.1086/688679. PMID:27792534.

Okamoto, K., and Kamezaki, N. 2014. Morphological variation in *Chelonia mydas* (Linnaeus, 1758) from the coastal waters of Japan, with special reference to the turtles allied to *Chelonia mydas agassizii* Bocourt, 1868. Curr. Herpetol. **33**: 46–56. doi:10.5358/hsj.33.46.

Owens, D.W., and Ruiz, G.J. 1980. New methods of obtaining blood and cerebrospinal fluid from marine turtles. Herpetologica. **36**: 17–20.

Parham, J.F., and Zug, G.R. 1996. *Chelonia agassizii* - valid or not? Mar. Turtle Newslett. **72**: 2–5.

Pilli, E., Casamassima, R., Vai, S., Virgili, A., Barni, F., D'Errico, G., et al. 2014. Pet fur or fake fur? A forensic approach. Invest. Genet. **5**: 7. doi:10.1186/2041-2223-5-7.

Pineda, O.G., and Rocha, A.R.B. 2016. Las tortugas marinas en México: Logros y perspectivas para su conservación. Soluciones Ambientales Itzeni A.C./Comisión Nacional de Áreas Naturales. Protegidas, México. ISBN.978-607-97436-0-4.

Pongratz, N., Gerace, L., and Michiels, N. 2002. Genetic differentiation within and between populations of a hermaphroditic freshwater planarian. Heredity, **89**: 64–69. doi:10.1038/sj.hdy.6800102. PMID:12080371.

Porter, T.M., and Hajibabaei, M. 2018. Over 2.5 million COI sequences in GenBank and growing. PLoS ONE, **13**: e0200177. doi:10.1371/journal.pone.0200177. PMID:30192752.

Rambaut, A., 2010. FigTree v1.3.1. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh. Available from https://github.com/rambaut/figtree/releases [accessed 19 January 2021]

Ratnasingham, S., and Hebert, P.D.N. 2007. BOLD: The Barcode of Life Data system. Mol. Ecol. Notes, **7**: 355–364. doi:10.1111/j.1471-8286.2007.01678.x. PMID:18784790.

Reid, B.N., Le, M., McCord, W.P., Iverson, J.B., Georges, A., Bergmann, T., et al. 2011. Comparing and combining distance-based and character-based approaches for barcoding turtles. Mol. Ecol. Resour. **11**: 956–967. doi:10.1111/j.1755-0998.2011.03032.x. PMID:21635698.

Reusche, M.R. 2020. Genetic structure of Green Sea Turtle (*Chelonia mydas*) foraging aggregations on the east coast of Florida. Honors undergraduate theses, University of Florida. 750. Available from https://stars.library.ucf.edu/honorstheses/750 [accessed online 2021-01-18].

Roden, S.E., and Dutton, P.H. 2011. Isolation and characterization of 14 polymorphic microsatellite loci in the leatherback turtle (*Dermochelys coriacea*) and cross-species amplification. Conservation Genet. Resour. **3**: 49–52. doi:10.1007/s12686-010-9284-4.

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E., and Sánchez-Gracia, A. 2017. DnaSP 6: DNA sequence polymorphism analysis of large datasets. Mol. Biol. Evol. **34**: 3299–3302. doi:10.1093/molbev/msx248. PMID:29029172.

SEMARNAT. 2019. Available from http://www.dof.gob.mx/normasOficiales/4254/semarnat/semarnat.htm.

Seminoff, J.A., Wallace, B.P., and Pritchard, P.C.H. 2013. Sea turtles of the eastern Pacific: advances in research and conservation. University of Arizona Press. Vol. 88. doi:10.1086/673833.

Silver-Gorges, I., Koval, J., Rodriguez-Zarate, C.J., Paladino, F.V., and Jordan, M. 2020. Large-scale connectivity, cryptic population structure, and relatedness in Eastern Pacific Olive ridley sea turtles (*Lepidochelys olivacea*). Ecol. Evol. **10**(16): 8688–8704. doi:10.1002/ece3.6564. PMID:32884651.

Tikochinski, Y., Carreras, C., Tikochinski, G., and Vilaça, S.T. 2020. Population-specific signatures of intra-individual mitochondrial DNA heteroplasmy and their potential evolutionary advantages. Sci. Rep. **10**(1): 211. doi:10.1038/s41598-019-569186. PMID:31937820.

Trivedi, S., Aloufi, A.A., Sankar, A.A., and Ghosh, S.K. 2016. Role of DNA barcoding in marine biodiversity. Saudi J. Biol. Sci. **23**: 161–171. doi:10.1016/j.sjbs.2015.01.001. PMID:26980996.

Vargas, S.M., Araújo, F.C., and Santos, F.R. 2009. DNA barcoding of Brazilian sea turtles (Testudines). Genet. Mol. Biol. **32**: 608–612. doi:10.1590/S1415-47572009005000050. PMID:21637526.

Vargas, S.M., Jensen, M.P., Ho, S.Y., Mobaraki, A., Broderick, D., Mortimer, J.A., et al. 2016. Phylogeography, genetic diversity, and management units of hawksbill turtles in the Indo-Pacific. J. Hered. **107**(3): 199–213. doi:10.1093/jhered/esv091. PMID:26615184.

Vásquez-Carrillo, C., Noriega-Hoyos, C.L., Hernandez-Rivera, L., Jáuregui-Romero, G.A., and Sullivan, S.K. 2020. Genetic diversity and demographic connectivity of Atlantic green sea turtles at foraging grounds in northeastern Colombia, Caribbean Sea. Front. Mar. Sci. **7**: 96. doi:10.3389/fmars.2020.00096.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., and Hebert, P.D.N. 2005. DNA barcoding Australia's fish species. Philos. Trans. R Soc. B Biol. Sci. **360**: 1847–1857. doi:10.1098/rstb.2005.1716. PMID:16214743.

Ward, R.D., Hanner, R., and Hebert, P.D.N. 2009. The campaign to DNA barcode all fishes, FISH-VBOL. J. Fish Biol. **74**: 329–356. doi:10.1111/j.1095-8649.2008.02080.x. PMID:20735564.

Xia, W., Zhang, B., Xing, D., Li, Y., Wu, W., Xiao, Y., et al. 2019. Development of high-resolution DNA barcodes for *Dioscorea* species discrimination and phylogenetic analysis. Ecol. Evol. **9**: 10843–10853. doi:10.1002/ece3.5605. PMID:31624585.

Yang, F., Ding, F., Chen, H., He, M., Zhu, S., Ma, X., et al. 2018. DNA barcoding for the identification and authentication of animal species in traditional medicine. Evid. Based Complement. Alternat. Med. **2018**: 1–18. doi:10.1155/2018/5160254. PMID:29849709.

Zahiri, R., Lafontaine, J.D., Schmidt, B.C., deWaard, J.R., Zakharov, E.V., and Hebert, P.D.N. 2017. Probing planetary biodiversity with DNA barcodes: The Noctuoidea of North America. PLoS ONE, **12**: e0178548. doi:10.1371/journal.pone.0178548. PMID:28570635.

Zhang, Z.P., Wang, X.Y., Zhang, Z., Yao, H., Zhang, X.M., Zhang, Y., and Zhang, B.G. 2019. The impact of genetic diversity on the accuracy of DNA barcoding to identify species: A study on the genus *Phellodendron*. Ecol. Evol. **9**: 10723–10733. doi:10.1002/ece3.5590. PMID:31624576.