Connectivity, population structure, and conservation of Ecuadorian green sea turtles

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ABSTRACT: Studies of highly migratory species that increase our understanding of the dynamics of genetic diversity, migratory routes, and genetic connectivity are essential for informing conservation actions. Genetic data for green turtles *Chelonia mydas* from Ecuador have only been available from Galápagos Islands (GPS) rookeries, but not from foraging aggregations. Furthermore, green turtles from habitats associated with mainland Ecuador (Machalilla National Park; MNP) have not been sampled. To assess the genetic relationships between nesting and foraging aggregations from these 2 regions and other regional populations, the mitochondrial DNA (mtDNA) control region was sequenced from 133 turtles. Conventional F_{ST} (haplotype frequency) and Φ_{ST} (sequence-based) values were low and non-significant between Ecuadorian rookeries, suggesting high connectivity between these sites located ca. 1000 km apart. Mixed stock analysis (MSA) indicated a dominant (>94%) GPS-MNP contribution to both foraging grounds, with small and nearly negligible contributions from other rookeries in the region (e.g. Costa Rica and Mexico). While orphan haplotypes were not included in the MSA because their rookery of origin is not known, their close genetic relationships to Western and Central Pacific mtDNA clades suggests that a relatively large percentage of turtles at the combined foraging sites (>10%) have been involved in transoceanic migration events. The genetic links between GPS and MNP *C. mydas* nesting populations revealed by our study highlight the need to incorporate the nesting populations from coastal Ecuador in more comprehensive future conservation planning.

KEY WORDS: *Chelonia mydas* · Galápagos · Machalilla · Connectivity · Ecuador · Conservation · Mixed stock analysis · Phylogenetics

INTRODUCTION

Phylogeographic studies of highly vagile species are important to an understanding of broad patterns of dispersal, as well as to identify important regions for the species' conservation. The use of genetic information provides a comprehensive account of population structure, connectivity, and overall demographic patterns of populations (Bowen & Karl 2007, Seminoff et al. 2008, Shamblin et al. 2012, Joseph et

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al. 2016). Obtaining genetic information, however, is particularly challenging in marine environments where physical barriers for gene flow are less evident and dispersal potential is high (Ward et al. 1994). Geographically dispersed populations can impose logistical and financial limitations on sufficient sampling of representative breeding and feeding populations. For instance, the circumtropical green turtle *Chelonia mydas* has the widest and most continuous distribution of all hard-shelled sea turtle species (Vieyra 2006). Previous work on *C. mydas* shows that regional genetic structure has been shaped by both intrinsic (behavior and ecology) and extrinsic (geographic, climate, and oceanography) events (Seminoff et al. 2008, Roden et al. 2013, Dutton et al. 2014a, Hamabata et al. 2014). For example, current levels of genetic diversity are potentially the result of glacial and inter-glacial paleo-oceanic periods that created opportunities for the establishment of new migratory routes (Formia et al. 2006, Hamabata et al. 2014). Genetic differentiation between Pacific and Atlantic basin populations is most likely the consequence of the formation of the Isthmus of Panama that imposed a barrier to gene flow between these oceans (Bowen et al. 1992, Bowen & Karl 2007, Naro-Maciel et al. 2008, Wallace et al. 2010, Duchene et al. 2012). Defining genetic clusters of green turtles across their broad geographic range has allowed delineation of Management Units (MUs) for conservation in several regions (Moritz 1994, Dethmers et al. 2006, Dutton et al. 2014a,b) within broader geographic regional MUs (see Wallace et al. 2010). Nonetheless, many green turtle rookeries along the coast of the southeast Pacific Ocean remain understudied, which limits both our understanding of evolutionary pathways and the identification of potentially new MUs.

The life history of the green turtle involves an early oceanic epipelagic phase lasting several years (Carr 1987) followed by a less vagile stage that is associated with neritic foraging grounds (Musick et al. 1997, Luschi et al. 2003) that commonly host individuals from a mixture of rookeries (Moncada et al. 2006, Bowen & Karl 2007). Green turtles show philopatry (Mayr 1963) and eventually return to their natal beaches, which can be hundreds or thousands of kilometers from their foraging areas, for reproduction (Lohmann et al. 1997, Bolten 2003). Reproductive stocks can be identified through the use of mitochondrial DNA (mtDNA) sequences (Bowen et al. 1992). Moreover, given the genetic distinctiveness of nesting stocks, their relative contributions to a given foraging ground can be estimated with mixed stock analysis (MSA) (Bolker et al. 2003). The MSA method

has the advantage over the traditional mark-recapture techniques in that it can be applied to individuals belonging to both genders and all size classes (Jensen 2010, Jensen et al. 2013, 2016), can involve modest sample sizes, and requires only a relatively short period of time to complete (Read et al. 2014). Despite numerous studies using MSA in marine turtles (Bass & Witzell 2000, Engstrom et al. 2002, Dutton et al. 2008, Jensen 2010, Nishizawa et al. 2013, Jensen et al. 2016, Joseph et al. 2016), important sampling gaps from Eastern Pacific (EP) populations remain, including the breeding populations nesting on mainland Ecuador. Furthermore, limited information exists on the levels of connectivity between foraging and nesting habitats, with only a few MSAs for the region (Amorocho et al. 2012, Heidemeyer 2015).

We examined a previously unstudied rookery of green turtles from the Pacific coast of mainland Ecuador at Machalilla National Park (MNP) in order to fill a void of genetic information from that region. The MNP contains the largest reported breeding population of green turtles along the coast of Ecuador, with the greatest number recorded at Isla de la Plata (ca. 470 total nests reported from nesting seasons 2008 to 2010) and fewer nests per area recorded from coastal sites at Drake Bay (n = 73 nests) and La Playita (n = 7 nests) (Peña-Mosquera 2010, Anhalzer et al. 2012). Unfortunately, green turtles at MNP face important conservation challenges (e.g. presence of feral dogs, illegal nest harvesting, urbanization, light pollution, beach motor traffic, garbage) as do olive ridleys *Lepidochelys olivacea* and hawksbill turtles *Eretmochelys imbricata* that also nest in this area (Peña-Mosquera 2010, Mast et al. 2016). Previous genetic work in Ecuador focused solely on females nesting on the Galápagos Islands (Dutton et al. 2014a, Naro-Maciel et al. 2014) but did not assay the foraging aggregations in the waters off these islands. Galápagos rookeries (GPS) apparently constitute a single individual genetic stock (the Galápagos MU; Dutton et al. 2014a) that has very limited connectivity to other rookeries across the EP. Some rookeries that are separated by a distance of approximately 500 km or more are commonly considered independent genetic stocks (Dethmers et al. 2006, Browne et al. 2010, Dutton et al. 2014a, Shamblin et al. 2015). Thus the distance between MNP and GPS rookeries (ca. 1000 km) would suggest that these populations should be genetically independent (i.e. distinguishable genetic stock and possibly new MU), although no genetic data are currently available that confirm a lack of connectivity between the rookeries.

In this study, we used mtDNA sequences to (1) determine the genetic relationships and connectivity between green turtle rookeries in MNP and GPS, (2) evaluate genetic composition at foraging grounds in MNP and GPS, (3) estimate the origin of orphan haplotypes (i.e. unknown rookery of origin) from phylogenetic trees, and (4) assess the extent of the Galápagos MU in the Southeast Pacific.

MATERIALS AND METHODS

Study area and data collection

Samples were collected from both nesting and foraging aggregations along continental-coastal Ecuador at MNP, Manabí province, western Ecuador. The MNP (01° 31′ 00″ S, 80° 42′ 00″ W) has a land area of ca. 551 km² and extends 2 nautical miles (ca. 3.7 km) along the coast and around Isla de la Plata (Peña-Mosquera et al. 2009). Isla de la Plata (1° 16′ 55″ S, 81° 3′ 84″ W) is a 14 km² continental island located 40 km from mainland Ecuador and known for presenting the highest concentrations of nesting green

turtles along the coast of Ecuador (Peña-Mosquera et al. 2009, Peña-Mosquera 2010) and the Pacific coast of South America (Mast et al. 2016). Nesting aggregations were sampled at beaches of La Playita on the mainland and of Isla de la Plata, and foraging aggregations were sampled around Isla de la Plata and off the coast of Puerto López on the mainland. Samples from GPS were collected from foraging aggregations around San Cristóbal, Española, Floreana, Darwin, and Wolf Islands (Fig. 1).

We obtained tissue samples (skin biopsies) for DNA extraction from a total of 133 green turtles: 43 from foraging aggregations at Puerto López and Isla de la Plata, herein considered as a single foraging aggregation given their geographic proximity (Fig. 1), 29 from nesting aggregations at MNP beaches (7 from La Playita and 22 from Isla de la Plata), and 61 from different foraging aggregations in the Galápagos Islands. Turtles were captured by direct swim-and-catch or while monitoring reproductive females during nesting activities at beaches. Morphometric measurements were taken with a flexible measuring tape: curved carapace length (CCL), curved carapace width (CCW), and total tail length (TTL) as per standard methods. We used a power hang scale (Do-All Outdoors) to obtain mass (± 0.5 kg) of turtles at the foraging grounds. Individuals were uniquely marked using Iconel tags placed on flippers, thereby avoiding pseudoreplication that would result from duplicate sampling of individuals. Turtles sampled at foraging grounds were returned to the site of capture. Because the sex of an immature green turtle cannot be determined with confidence on the basis of an external examination (Bolten & Bjorndal 1992), we used a cut-off size of 70 cm CCL to discriminate immature from mature individuals (Amorocho et al. 2012). This is consistent with the mean nesting size of reproductive females at GPS, where 2 modal values of 80 and 95 cm CCL have been recorded, with the smallest nesting female documented for the archipelago at 60.7 cm CCL (Zárate 2002). The sex of green turtles with CCL >70 cm was determined based on sexual dimorphism presented in *Chelonia mydas* (Wibbels 1999, Wyneken et al. 2013). Thereafter,
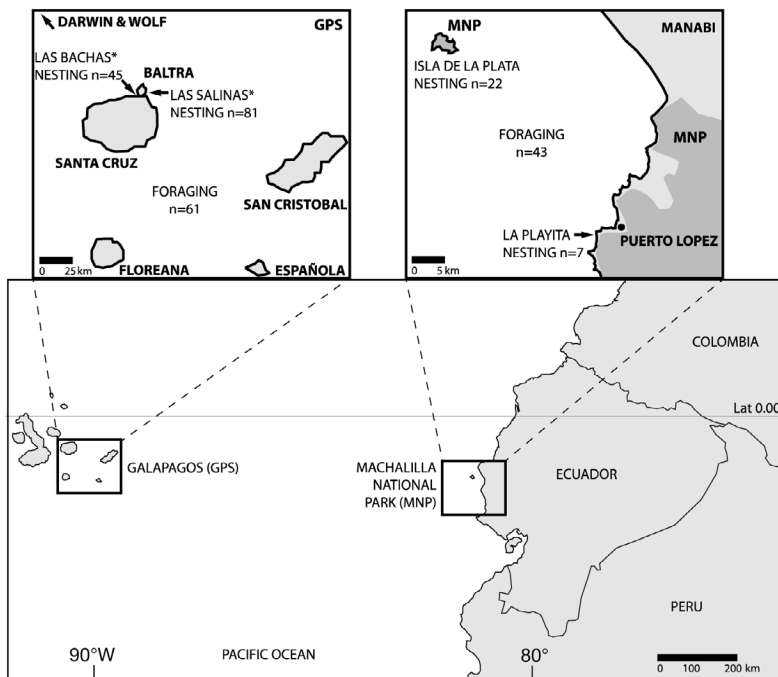

Fig. 1. Location of study sites in Ecuador. Boxes correspond to Galápagos (GPS) and Machalilla National Park (MNP) sampling locations, separated by ca. 1000 km. Gray area in MNP corresponds to the protected zone of the park (including Isla de la Plata, located ca. 40 km from the mainland). Names with nesting and foraging numbers represent locations and number of individuals sampled. Names with asterisks correspond to sites sampled by Dutton et al. (2014a) used in this study

mature adult males were categorized as individuals presenting tail measurements of 25 cm (or longer) (Hamann et al. 2006). Skin biopsies were preserved in 90% ethanol in the field, and stored at −80°C. All of the sequences for GPS rookeries incorporated into this study (n = 126: 45 from Las Bachas on Santa Cruz Island and 81 from Las Salinas on Baltra Island) were downloaded from GenBank and correspond to the work by Dutton et al. (2014a).

Laboratory analysis

Mitochondrial DNA (mtDNA) was isolated from tissue using a Qiagen DNeasy® Blood & Tissue Kit following the manufacturer's protocol, and DNA quality and concentration were measured with Nanodrop 1000 (ThermoScientific). Primers LTEi9 (5′-GAA TAA TCA AAA GAG AAG G-3′) and H950 (5′-GTC TCG GAT TTA GGG GTT T-3′) (Abreu-Grobois et al. 2006) were used in a polymerase chain reaction (PCR) to amplify a ~832 bp fragment of the control region. PCR was performed in 25 to 50 μl total volumes with cocktail concentrations of 1× buffer, 1.25 mM of each deoxynucleoside triphosphate (dNTPs), 1.5 mM $MgCl_2$, 10 μM of each primer, 1.25 U *Taq* polymerase, and ~40 ng of template DNA. Amplification was run under an initial denaturation at 94−96°C for 3 min, followed by 35 to 45 cycles of denaturation at 94−96°C for 30 s, annealing of primers at 55°C for 30 s, and extension of primers and elongation at 72°C for 10 min. To detect PCR products, 3 μl aliquots of product were run on 1% agarose gels stained with SYBR® Safe (Invitrogen) and visualized with Biorad Gel Doc XR (BIORAD) for photo-documentation. PCR products were purified with 20% ExoSap reactions following the manufacturer's protocol. All samples were sequenced by Macrogen (Seoul).

Statistical analysis

Sequences were aligned using Geneious Basic v.8.0.5 (www.geneious.com, Kearse et al. 2012) under default settings for Geneious Alignment against reference GenBank haplotypes (Table S1 in the Supplement at www.int-res.com/articles/suppl/n032p251 _supp.pdf). Novel haplotypes were assigned the standard nomenclature *CmP* for *C. mydas*, with a suffix added to denote the sampling locality of the haplotype (see Dutton et al. 2008, 2014a). Genetic comparisons between the Ecuadorian rookeries and foraging aggregations (GPS and MNP) were assessed based

on several estimates of genetic differentiation: haplotype frequencies, haplotype diversity (*h*), and nucleotide diversity (π). Population structure was tested by conducting analysis of molecular variance (AMOVA), pairwise F_{ST} comparisons, and pairwise exact test of population differentiation. Both sequence-based Φ_{ST} and conventional F_{ST} distance measures (10 000 permutations) (Reynolds et al. 1983) were used to calculate within- and among-population diversity in Arlequin v. 2.0 (Schneider et al. 2000). The same steps were taken to compare foraging aggregations in MNP and GPS. Because Western Pacific (WP) haplotypes were found frequently in the foraging aggregations, we replicated these analyses removing WP haplotypes.

Phylogenetic analysis

The best fitting model of evolution for the locus in the studied populations (TrN+I) was determined with jModelTest v.0.1.1 (Posada 2008) via Akaike's information criterion (AIC, Burnham & Anderson 2002). A phylogenetic reconstruction primarily to investigate the origin of several orphan haplotypes found in Ecuadorian foraging aggregations was conducted using Bayesian inference in MrBayes v.3.2 (Ronquist & Huelsenbeck 2003), with 2 runs of 4 simultaneous Markov chains, each for 10 million generations, and sampling of parameters and trees every 1000 generations. Convergence of the chains was confirmed using TRACER v.1.4 (Rambaut & Drummond 2007) and AWTY (Nylander et al. 2008), followed by a 25% burn-in of all retrieved trees to calculate posterior probabilities in a 50% majority-rule consensus tree. We used *Natator depressus* (GenBank accession no. U40662) as an outgroup (Duchene et al. 2012), as well as additional sequences from GenBank from EP rookeries that nested at Michoacán, Revillagigedos Islands, and Costa Rica, from Central Pacific (CP) rookeries in Hawaii (Dutton et al. 2014a, Heidemeyer 2015), and from WP populations nesting on American Samoa, the Republic of the Marshall Islands, and Ulithi Atoll-Yap (Dutton et al. 2014b). We also reconstructed a time-calibrated tree under a relaxed-clock framework in BEAST v.1.7.5 (Drummond & Rambaut 2007), after rejection of a global molecular clock (likelihood ratio test, Felsenstein 1981). The tree was calibrated using 0.01751 substitutions site^{-1} lineage^{-1} million yr^{-1} as the rate of evolution of the mtDNA control region of green turtles (Formia et al. 2006). Our analyses were performed for 10 million generations using a random starting tree (tree prior specia-

tion: Yule process). Stationarity, high effective sample sizes (ESS > 200), and 95% highest posterior density intervals (HPDs) were evaluated for all parameters in TRACER (Rambaut & Drummond 2007). A consensus tree with divergence times was obtained for unique green turtle sequences after discarding the first 25% of sampled trees. Finally, to represent intraspecific phylogenies and geographic distribution of genetic diversity of Ecuadorian haplotypes in relation to the EP region, a minimum-spanning network of absolute distances between mtDNA haplotypes was constructed using TCS v.1.2.2 (Clement et al. 2000) and Arlequin v. 2.0 (Schneider et al. 2000) using all published nesting haplotypes from Dutton et al. (2014a) (see Fig. 3).

MSA of Ecuadorian foraging populations

The contributions of distinct nesting aggregations to the MNP and GPS green turtle foraging grounds were estimated with Bayesian MSA using the program Bayes (Pella & Masuda 2001). Source haplotype frequencies were taken from published nesting stock characterizations from the EP sites Michoacán, Revillagigedos (Mexico), Nombre de Jesús (Costa Rica), and Galápagos, as well as the CP Hawaiian archipelago (Table S1). Given that one of the haplotypes sampled in the MNP has an identified origin in WP rookeries (*CmP22.1*; Dutton et al. 2014b), haplotypes from American Samoa, Marshall Islands, and Ulithi Atoll were also included in the MSA. The MSA was conducted using both uniform (flat: equal values for each stock) and informed (weighted: different values) priors (Table S2 in the Supplement). Using informed priors allows incorporating knowledge of the species to provide a stronger and more biologically meaningful result in cases where genetic structure is weak (Jensen et al. 2013). We used 'rookery population size' and 'geographic distance' from sources as weighted priors, as these are widely used in MSA and influence the estimated composition of the foraging grounds (Proietti et al. 2012). 'Geographic distance' prior was set for each stock as the distance from the putative stock to the foraging area, over the sum of distances from all stocks to the foraging area. These distances were obtained using Google Earth. 'Population size' prior was set as the proportion of the size of each stock to the total population size derived by the summation of all stock abundances included in the analysis (Table S2). For each of the 3 models (uniform, geographic distance, and rookery population size), a total of 50 000 Markov Chain Monte Carlo generations

were run for 8 chains from MNP foraging grounds and 5 chains from GPS foraging aggregations. Each of the chains was given a different starting point, and the number of chains corresponded to the potential source origins used for MNP and GPS (see above). Posterior distribution was calculated using a burn-in of 25 000 runs, and the shrink factor of Gelman & Rubin (1992) was calculated to test if chains had converged. We excluded individuals with haplotypes not found in any nesting rookery (orphan haplotypes) because they were uninformative.

RESULTS

Sequence diversity in Ecuador

Genetic analysis of the 133 Ecuadorian samples resolved a total of 14 haplotypes. From the 29 individuals sampled in the MNP rookery, only 4 haplotypes were recovered (*CmP4.1*, *CmP4.4*, *CmP4.6*, and *CmP4.7*), the same as those reported in GPS and other nesting individuals in the EP (Table 1) (Michoacán and Costa Rica; Dutton et al. 2014a). When combining our results with those from the previously published literature, these 4 haplotypes were found to be the most common in the Ecuadorian rookeries, occurring in 143 of 259 individuals for GPS beaches, and 79 of 259 individuals for both foraging grounds (Table 1). Only 3 haplotypes previously reported in GPS rookeries were not found in this study (see Dutton et al. 2008). One of the haplotypes found in nesting individuals in continental Ecuador (*Cmp4.1*) has also been reported in other foraging aggregations across the EP (Japan: Hamabata et al. 2014) and CP (Palmyra Atoll; Naro-Maciel et al. 2014). The other 10 haplotypes recovered in this study were found only at foraging aggregations: 3 only found in MNP, 4 only found in GPS, and 3 were shared between these 2 foraging populations (Table 1). Three out of the 10 haplotypes have also been previously reported for GPS rookeries (Dutton et al. 2014a). Interestingly, 1 individual sampled at the MNP foraging aggregations carried haplotype *CmP22.1*, which is known to occur at nesting rookeries in the CP (see below; Dutton et al. 2014a). Therefore, 6 out of the 10 haplotypes were categorized as 'orphan' as they have not been reported in any rookery, including 3 new haplotypes reported here for the first time (*CmP93.2*, *CmP4.19*, and *CmP112.1*; GenBank accession numbers KY350180, KX499513, and KY350181, respectively). Although *CmP112.1* and *CmP4.19* have been previously identified in foraging individuals in GPS

Table 1. mtDNA haplotype profiles in Ecuadorian green turtle *Chelonia mydas* habitats along with their GenBank accession numbers. Haplotype names follow conventional nomenclature. 'GPS nesting' column corresponds to Galápagos (GPS) rookeries data from Dutton et al. (2014a); sources for the other data are listed in the 'Reference' column. MNP: Machalilla National Park

Haplotype	MNP foraging	MNP nesting	GPS foraging	GPS nesting	Total	GenBank acc. no.	Reference
CmP4.1	11	3	13	26	53	KC306666	Dutton et al. (2014a)
CmP4.4	2	2	2	6	12	KC306665	Dutton et al. (2014a)
CmP4.6	18	12	16	51	97	KC306647	Dutton et al. (2014a)
CmP4.7	5	12	12	31	60	KC306660	Dutton et al. (2014a)
CmP4.9	0	0	0	1	1	KC306643	Dutton et al. (2014a)
CmP4.11	0	0	0	1	1	KC306661	Dutton et al. (2014a)
CmP4.15[a]	0	0	1	0	1	KX499506	Dutton et al. (2014a)
CmP15.1	0	0	1	3	4	KC306649	Dutton et al. (2014a)
CmP17.1	1	0	2	4	7	KC306648	Dutton et al. (2014a)
CmP24.1	2	0	0	1	3	KC306646	Dutton et al. (2014a)
CmP22.1	1	0	0	0	1	KF311747	Dutton et al. (2014b)
CmP93.1	0	0	0	2	2	FJ917194	Dutton et al. (2014a)
CmP93.2[b]	1	0	1	0	2	KY350180	This study
CmP97.1[a]	1	0	9	0	10	FJ917198	P. H. Dutton et al. (unpubl.)[a]
CmP94.1[a]	1	0	0	0	1	FJ917193	P. H. Dutton et al. (unpubl.)
CmP112.1[b]	0	0	3	0	3	KY350181	This study
CmP4.19[b]	0	0	1	0	1	KX499513	This study
Total	43	29	61	126	259		

[a]Orphan haplotypes reported in other foraging aggregations; [b]Novel orphan haplotypes reported here for the first time

(P. H. Dutton et al. pers. comm.) and Costa Rica (M. Heidemeyer unpubl.), these sequences have not previously been published. A fourth orphan haplotype, *CmP4.15*, recently reported in Gorgona Islands in Colombia (M. Sanchez pers. comm.), was found in GPS foraging aggregations. The last 2 orphan haplotypes in MNP and GPS foraging grounds (*CmP97.1* and *CmP94.1*) were reported as orphan in the CP (Palmyra Atoll: Naro-Maciel et al. 2014).

As mentioned previously, 4 haplotypes were the most dominant types across all habitats. At the MNP nesting aggregations, haplotypes *CmP4.6* and *CmP4.7* were present at high frequencies, each with 12 individuals out of the 29 sampled (41.3%). In GPS nesting individuals, the 3 haplotypes with the highest frequencies were *CmP4.6* (n = 51; 40.5%), *CmP4.7* (n = 31; 24.6%), and *CmP4.1* (n = 26; 20.6%) out of the 126 individuals analyzed by Dutton et al. (2014a). The MNP foraging aggregations were dominated by haplotypes *CmP4.6* and *CmP4.1* (n = 18; 41.8% and n = 11; 25.5%, respectively). Finally, GPS foraging individuals (n = 61) presented haplotypes *CmP4.6*, *CmP4.1*, and *CmP4.7* in similar frequencies (n = 16, 26.2%; n = 13, 21.3%; and n = 12, 19.6%, respectively).

The Ecuadorian nesting aggregation at MNP had a moderate h of 0.665 and low π of 0.002 (Table 2), indicating the presence of genetically similar haplotypes. In contrast, these estimates were substantially higher at both MNP and GPS foraging aggregations, where h was 0.749 and 0.831, respectively, indicating the abundance of haplotypes with origins other than Ecuadorian nesting grounds. Nucleotide diversity at the MNP foraging ground was moderate, with π = 0.004, and almost 4 times higher at the GPS foraging aggregation, with π = 0.013, which reflects the abun-

Table 2. Mitochondrial control region sequence diversity for Ecuadorian green turtles *Chelonia mydas*. Sample size (n), number of haplotypes (H), nucleotide diversity (π, with standard deviation, SD), and haplotype diversity (h, with SD) values are shown. Data for Galápagos (GPS) rookeries were taken from Dutton et al. (2014a). Values for GPS and Machalilla National Park (MNP) foraging aggregations (bottom row for each location) correspond to results without Western Pacific haplotypes

Location	n	H	π	SD	h	SD
MNP rookery	29	4	0.002	0.0014	0.665	0.0507
MNP foraging aggregations	43	10	0.004	0.0027	0.749	0.0511
	41	8	0.002	0.0017	0.731	0.0505
GPS rookeries	126	10	0.001	0.0010	0.734	0.0234
GPS foraging aggregations	61	11	0.013	0.0069	0.831	0.0225
	49	9	0.002	0.0014	0.773	0.0306

Table 3. Pairwise F_{ST}, Φ_{ST}, and Fisher's exact test for population differentiation between: (1) Galápagos (GPS) and Machalilla National Park (MNP) rookeries, (2) GPS and MNP foraging aggregations, and (3) GPS and MNP foraging aggregations without Western Pacific (WP) haplotypes (no WP). Significance values from Arlequin were at the 0.05 level

	F_{ST}	Φ_{ST}	Exact test
Rookeries	0.0073 (p > 0.05)	0.052 (p > 0.05)	0.738 (p > 0.05)
Foraging	0.0097 (p > 0.05)	0.025 (p > 0.05)	0.106 (p > 0.05)
Foraging (no WP)	0.014 (p > 0.05)	0.0643 (p = 0.018)	0.600 (p > 0.05)

dance of genetically distant haplotypes endemic to the WP (see below; Table 2). These values were much lower (π = 0.002) when we removed WP haplotypes from the analysis (Table 2).

Population structure

There was no significant genetic differentiation between the 2 Ecuadorian nesting populations (Table 3), suggesting genetic connectivity between GPS and MNP (F_{ST} = 0.0073, p > 0.05; Φ_{ST} = 0.052, p > 0.05). Foraging aggregations without WP haplotypes followed the same pattern as nesting populations of little genetic differentiation (F_{ST} = 0.0097, p > 0.05; Φ_{ST} = 0.025, p > 0.05), with most of the variation explained within populations (AMOVA). When WP haplotypes were incorporated into the analysis, only sequence-based Φ_{ST} distance measures were significant (F_{ST} = 0.014, p > 0.05; Φ_{ST} = 0.0643, p = 0.018), with a small proportion of variance (~5%) explained among groups.

Phylogenetic and phylogeographic analysis

To determine phylogenetic affinities and origins of new orphan haplotypes, the Bayesian reconstruction included the 17 haplotypes from Ecuador plus additional haplotypes from GenBank. Ecuadorian haplotypes from nesting individuals were nested within 2 previously recognized clades (Fig. 2): (1) CP-EP Clade (Dutton et al. 2014a) and (2) a WP clade (Dethmers et al. 2006, Naro-Maciel et al. 2014). The CP-EP clade includes 3 subclades with samples from Hawaii, Revillagigedo, Michoacán, Costa Rica, Galápagos (Dutton et al. 2014a), and MNP in coastal Ecuador. The lack of support in several clusters within the CP-EP clades failed to recover the monophyletic clades as reported by Dutton et al. (2014a). Nevertheless, the Bayesian tree supports the genetic

affinity of *Cmp4.19* (Dutton et al. 2014a) and *Cmp4.15* from foraging aggregations from GPS to haplotypes *CmP93.2* and *CmP4.6* (Dutton et al. 2014a) from rookeries at GPS, MNP, Michoacán, and Costa Rica. Although haplotype *Cmp4.6* was found in the EP Clade III by Dutton et al. (2014a), our phylogenetic reconstruction placed it within samples that correspond to EP Clade II. Surprisingly, 3 haplotypes found in GPS and MNP foraging aggregations (*CmP97.1*, *CmP112.1*, and *CmP22.1*) corresponded to haplotypes found in CP and WP. Haplotype *CmP22.1* has been found in nesting rookeries from American Samoa, the Marshall Islands, and Ulithi Atoll (Dutton et al. 2014b), while the orphan haplotypes *CmP97.1* and *CmP112.1* are phylogenetically related to WP rookeries (Fig. 2) (Dutton et al. 2014a, Naro-Maciel et al. 2014, P. H. Dutton unpubl.). The last 3 haplotypes were found in 19.7% of GPS foraging grounds and in 4.6% of the MNP foraging aggregation.

The Bayesian calibrated tree supports the origin of green turtles in the Pliocene ca. 4.40 million yr (HPD = 2.70–6.37; stem-based estimate) falling within the range proposed using whole mitogenone calibration (Duchene et al. 2012). Our tree also suggests a young origin (<0.1 million yr) for all haplotypes found in Ecuadorian rookeries.

The haplotype network of Ecuadorian rookeries (MNP and GPS; Fig. 3) showed most nesting females bearing the aforementioned common haplotypes (*CmP4.6*, *CmP4.7*, and *CmP4.1*; see also Table 1), and few individuals characterized by less common haplotypes that had connections of 1 bp in a star-like fashion. The 3 common haplotypes were also commonly found in several individuals from Michoacán and Revillagigedo when pooled with data from GenBank (see Dutton et al. 2014a).

MSA

MSA was performed using only haplotypes with known origin. Because MNP and GPS nesting populations were not significantly different (population genetic results), these 2 stocks were pooled into a single GPS-MNP entity for subsequent analyses. The MSA was run using uniform and weighted priors (population size, distance from source rookery); however, weighted priors are only useful for estimating source contributions when the estimates using uni-
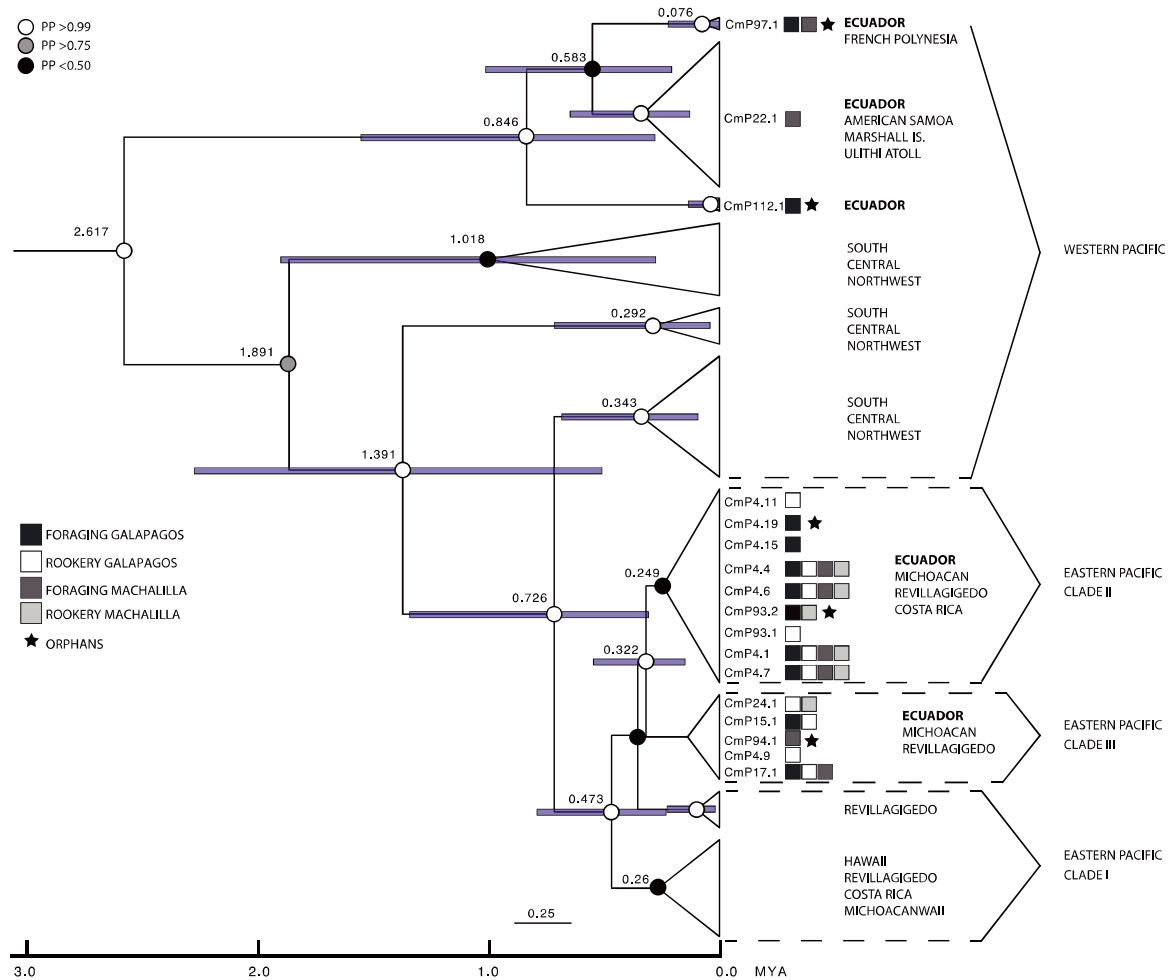
Fig. 2. Phylogenetic reconstruction of green turtle *Chelonia mydas* mitochondrial DNA control region haplotypes combining sequences from this study and those from Dutton et al. (2014a). Only haplotypes found in Ecuador are labeled with standard nomenclature. Boxes with different shading next to haplotypes indicate their presence in foraging aggregations in the GPS (black squares) or MNP (dark grey squares), or in rookeries in GPS (white squares) or MNP (light grey squares). Orphan haplotypes are marked with a star. Mean marginal means for age estimates are shown as blue bars on nodes. Scale at bottom is in millions of years. Haplotype names follow Table 1. Terminal branches were collapsed (triangles) and outgroups removed for better visualization. PP: posterior probability

form priors (based on stock haplotype frequencies only) fail to provide explanatory results. In our case, the uniform prior provided a reasonable estimate on the contributions by the major stocks to the analyzed foraging grounds, on which we focus hereafter. The estimated contributions indicate a predominance of the combined GPS-MNP stock to both foraging grounds with average contributions of around 96.5% (95% to GPS and 98% to MNP; Table 4, Fig. S1 in the Supplement at www.int-res.com/articles/suppl/n032p251_supp.pdf). The Michoacán source appeared to

Table 4. Mixed stock analysis estimated contributions of Pacific green turtle *Chelonia mydas* stocks to Machalilla National Park (MNP) and Galápagos (GPS) foraging aggregations. Results are shown for flat-prior analysis only (mean and 95% confidence intervals are indicated). Estimated contributions >5% are shown in **bold**; blank cells indicate no data

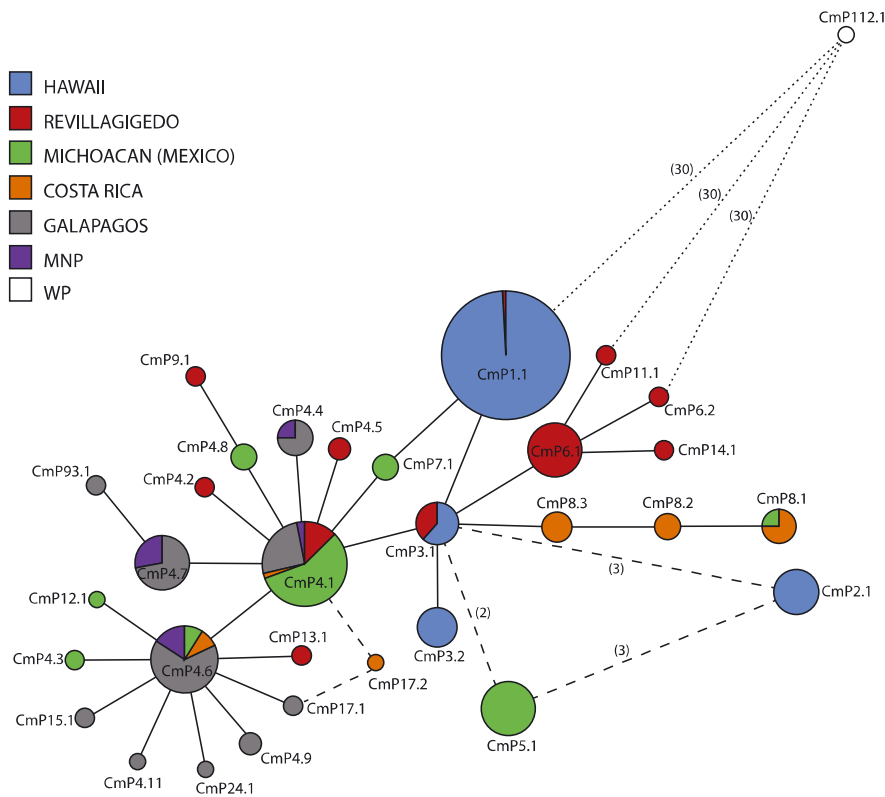| Stock	MNP		GPS	
	Mean	CI (95%)	Mean	CI (95%)
Revillagigedo (Mexico)	0.41	0.00–3.71	0.56	0.00–4.14
Michoacán (Mexico)	1.02	0.00–**8.83**	1.26	0.00–**9.29**
Nombre de Jesús (Costa Rica)	0.77	0.00–**6.72**	0.78	0.00–**5.95**
GPS-MNP	**94.89**	**72.95–96.64**	**96.98**	**87.33–99.97**
Hawaii	0.69	0.00–2.84	0.42	0.00–3.15
American Samoa	1.16	0.00–**11.15**		
Ulithi Atoll	1.16	0.00–**10.96**		
Marshall Islands	1.13	0.00–**10.68**		

Fig. 3. Haplotype network of nesting green turtle *Chelonia mydas* mitochondrial control region haplotypes found in the Eastern Pacific. Each circle corresponds to a unique haplotype, the size is proportional to the number of sampled individuals carrying that haplotype over all data used (this study + Dutton et al. 2014a), and the connections represent 1 base pair (bp) difference unless specified otherwise (number in parentheses). Dashed lines correspond to alternative haplotype connections, and dotted lines depict connections of 30 bp. Haplotype names reported in Ecuador follow Table 1. MNP: Machalilla National Park, WP: Western Pacific

provide a small contribution of 1.3 and 1.0% to GPS and MNP, respectively. For MNP, a minimal contribution of 1.2 and 1.1% was estimated to come from American Samoa and Micronesia (Ulithi Atoll and Marshall Islands), respectively. The estimated contributions to both Ecuadorian foraging grounds from Revillagigedo Archipelago, Nombre de Jesús (Costa Rica), and Hawaiian rookeries were negligible (mean of ≤1.0%). Given that, with the exception of the Ecuadorian sources, all estimates include 0 within their confidence limits, it is likely that only the Ecuadorian rookeries contribute to the foraging sites under study (Table 4).

Morphological diversity of foraging individuals

Size class histograms show a substantial number of immature individuals in both foraging grounds. Based on the cut-off size of 70 cm to distinguish immature from mature individuals (see 'Materials and methods'), we recorded a total of 18 immature/juveniles (<70 cm CCL) and 43 adults (>70 cm CCL), of which 20 were females and 23 males (Fig. S2 in the Supplement). On the other hand, MNP foraging individuals were dominated by immature/juvenile turtles (n = 26). Remaining adult individuals sampled were 11 females and 6 males (Fig. S2). In both foraging aggregations, all WP haplotype-bearing individuals (*CmP 97.1, CmP 112.1,*

and *CmP 22.1*) (Fig. S2) corresponded to turtles with yellow morphotypes; all 4 immature/juvenile individuals for GPS (mean CCL = 53.75 cm, range = 44−76) and 1 female in MNP (CCL = 73 cm). Nesting females in MNP presented a mean CCL of 86.52 cm (n = 27; SD = 5.25 cm, range = 77.3−98.0 cm).

DISCUSSION

Rookeries

The genetic similarity found between coastal Ecuador (MNP) and Galapagos rookeries (GPS) suggests that these 2 nesting assemblages are behaving as a single panmictic stock. Both nesting sites share unique haplotypes (*CmP4.4* and *CmP4.7*), and shared haplotypes (*CmP4.1* and *CmP4.6*) found in green turtle nesting beaches across the EP (Table S1: Hawaii, Michoacán, and Costa Rica). Although green turtle rookeries that are more than 500 km apart may behave as independent stocks (Dethmers et al. 2006, Bowen & Karl 2007, Dutton et al. 2014b, Shamblin et al. 2015), the GPS and MNP rookeries are genetically similar despite the fact that they are about 1000 km apart. However, our results were not unexpected given that a similar pattern was found between GPS and Mexican rookeries using nuclear microsatellites and mtDNA data over larger distances than those pre-

sented here (Dethmers et al. 2006, Dutton et al. 2008). We hypothesize that this finding might be explained by at least 3 scenarios: (1) high levels of ongoing gene flow between GPS and MNP females, (2) recent colonization of nesting females to either of these beaches with little time for genetic differentiation (i.e. evolutionary connectivity), or (3) insufficient resolution using currently available genetic markers. Although none of these were explicitly tested here, our time-calibrated tree coincides with a very recent origin for the region's haplotypes (< 0.1 million yr). Furthermore, the star-like haplotype network for MNP and GPS rookeries (Fig. 3) revealed little genetic structure. This result is indicative of recent shared ancestry or recent divergence of several endemic haplotypes separated by 1 bp from more common and widely distributed haplotypes, ultimately suggesting recent colonization (see Avise 2000). A common limitation of the use of mtDNA in marine turtle studies in general is the difficulty to detect population structure at fine scales (reviewed by Bowen & Karl 2007).

We recognize that a more accurate estimate of gene flow between highly similar rookeries from MNP and GPS could require a combination of both nuclear and mitochondrial DNA (bi-parental and female-mediated gene flow) markers. Alternatively, the use of mitochondrial short tandem repeats (Tikochinski et al. 2012) or mitogenomic sequencing could help reveal cryptic differentiation among Ecuadorian populations, as found in Caribbean rookeries (Shamblin et al. 2012). Despite the limitations, these are robust results which suggest close genetic links between the Ecuadorian stocks in spite of their geographic separation that could be used as the first step in redefining MUs for these populations. At a regional scale, and besides the sharing of common haplotypes between MNP and GPS, the haplotype network showed that MNP nesting females shared haplotypes with individuals breeding in Mexico and Costa Rica (*CmP4.6* and *CmP4.7*; Fig. 3). The MNP is apparently an important nesting area for green turtles that share genetic ties with other rookeries along the EP. Thus, our study expands the geographic distribution of these haplotypes in the EP to the newly sampled MNP beaches.

Foraging grounds

Foraging grounds at MNP and GPS exhibited relatively high π and h diversity, characteristic of habitats with a mixture of diverse local haplotypes (high h) with highly divergent haplotypes (high π). The largest difference in nucleotide diversity between GPS and MNP foraging grounds (GPS π = 0.014 vs. MNP π = 0.005) clearly reflects this pattern where apparent WP haplotypes were more abundant in GPS waters (18%) than in MNP (< 5%) (Table S2). Furthermore, only the nucleotide-based measure of genetic distance (and not haplotype frequency measures) was significant between these regions, consistent with the presence of highly divergent haplotypes found in GPS foraging individuals. The observed differences were non-significant when removing these WP haplotypes from the analysis, which is clear evidence of their influence inflating values of π, thus increasing the genetic composition of the foraging population in GPS. The orphan haplotype *CmP97.1*, closely related to foraging haplotypes from Palmyra Atoll (Naro-Maciel et al. 2014), was found in 12 of 61 (19.67%) turtles from GPS foraging grounds, and was previously also reported from foraging grounds off Gorgona Island in Colombia (Amorocho et al. 2012). The orphan and new haplotype *CmP112.1*, which is phylogenetically related to WP rookeries, was found at much lower frequency (4.91%, 3 out of 61) in the GPS foraging grounds (2 immature and 1 mature female; Fig. S2). While their exact origins remain unknown, Ecuadorian individuals carrying these 2 orphan haplotypes exhibit the 'yellow morphotype' characteristic of WP green turtles (see Zárate 2012), which offers a possible clue to their origins. The apparent orphan haplotypes, along with the previously reported *CmP22.1* from American Samoa, Marshall Islands, and Ulithi Atoll (Dutton et al. 2014b), represent evidence for trans-Pacific associations, suggesting that trans-oceanic links among Pacific green turtles are more widespread than previously thought for the species but not unique among sea turtles (*Caretta caretta*: Bowen et al. 1995, *Dermochelys coriacea*: Dutton et al. 2000). A simple visualization of passive drift movements by oceanic currents (adrift.org.au) from putative CP and WP sites provide possible routes to EP regions (e.g. GPS, MNP, and Gorgona; Amorocho et al. 2012). Thus, dispersal of individuals carrying WP haplotypes could be facilitated towards eastern regions either via the South Equatorial current in combination with the Humboldt Current from the south, or the North Equatorial current in combination with El Niño from the north. Previous satellite telemetry studies (*C. caretta*, *D. coriacea*, *Eretmochelys imbricata*: Mast et al. 2016) and tag recovery from hatchlings captured in longline fisheries (*C. caretta*; Boyle et al. 2009) have identified the importance of these dynamic oceanic currents in sea turtle movements. Thus, these currents could not only explain the presence of trans-Pacific haplotypes in

the EP, but could also be responsible for the genetic patterns of connectivity reported here between GPS and MNP at a much smaller geographic scale.

In a regional comparison, and excluding divergent WP haplotypes from our analyses, the nucleotide diversity in GPS foraging grounds ($\pi = 0.002$) is within the ranges found at other previously studied foraging aggregations, such as the ones in CP (0.002–0.003, Hawaii; Dutton et al. 2008), Caribbean (0.003, Costa Rica; Bjorndal et al. 2005), and Atlantic (0.002–0.006, Brazil; Naro-Maciel et al. 2006). However, when incorporated, the WP haplotypes inflate this population genetic metric ($\pi = 0.013$), placing the GPS aggregation within much higher ranges like those found at EP foraging aggregations (0.011, Colombia; Amorocho et al. 2012). This result highlights the importance of GPS foraging grounds contributing to the genetic diversity of green turtles in the EP. One such case is the orphan *CmP4.15*, a rare haplotype found in 1 individual in GPS foraging grounds and phylogenetically linked to haplotypes found in rookeries from Ecuador and Mexico. Recent reports from Gorgona Island in Colombia have found this same haplotype in other foraging individuals (M. Sanchez pers. comm.), thus indicating a broad distribution regardless of its source. Further sampling on rookeries along the EP coast of South, Central, and North America will be important to identify its origin. Furthermore, the number of orphan haplotypes clustering within EP clades reported here (*CmP4.19*, *CmP93*.2 about 3% of all foraging turtles) and elsewhere (*CmP94.1*; Dutton et al. 2014a) could suggest either the presence of many genetically uncharacterized nesting stocks in the EP region, or the presence of rare local haplotypes that have not been detected due to small sample sizes studied at rookeries.

MSA

Our MSA results showed a predominance of a GPS-MNP contribution to both foraging grounds (93.8% to GPS and 94.1% to MNP), revealing a very high degree of connectivity between rookery and foraging grounds in Ecuador. High connectivity among regional green turtles has also been documented within the Hawaiian MU, where most of the haplotypes found in foraging aggregations have their origins at proximate Hawaiian rookeries (Dutton et al. 2008). Extremely low contributions to Ecuadorian foraging grounds from regional stocks (e.g. Costa Rica and Mexico), could suggest a pattern for many foraging grounds along the EP composed mostly of local MUs. On the other hand, foraging grounds in this study are found at the easternmost edge of the geographic range for green turtles in the EP, which may explain the limited exchange with other rookeries. Nevertheless, this pattern does not exclude the possibility that individuals from local rookeries may feed at multiple, common foraging grounds. For example, foraging turtles from GPS are mostly from rookeries in GPS, but turtles from GPS rookeries could also use foraging grounds in GPS, MNP, and Gorgona Island and coastal areas in Central America, as supported by post-nesting satellite-tacked individuals from GPS (Seminoff et al. 2008). This is consistent with their life histories, since no breeding takes place at foraging habitats. The lack of genetic differentiation combined with the MSA results supports a high connectivity between MNP and GPS, and suggests the existence of an important marine corridor connecting these 2 regions in Ecuador. Amorocho et al. (2012) found that a high percentage of Gorgona green turtles in Colombia have their origins in GPS nesting beaches. These findings together provide evidence for yet another axis of marine gateways in this region with important conservation implications.

Implications for management and conservation

Our study contributes information on the genetic diversity and connectivity of green turtles found in an important region of Ecuador (MNP) that had been unstudied. The existing data from GPS rookeries and newly published breeding haplotypes from MNP could be used to reassess the composition of green turtles in the southern range of the EP. The evidence implies that green turtles born in GPS utilize MNP as foraging grounds and vice versa, and hence conservation efforts in both areas are required to protect these 2 genetic stocks. On the basis of the present results, it would be justifiable to extend the previously defined Galápagos MU (Dutton et al. 2014a) to include the continental Ecuador rookeries (MNP). A series of pervasive anthropogenic threats including loss of habitat, coastal development, effect of fishery by-catch, poaching, boat strikes, and climate change are thought to determine marine turtle survival in the region. Although both nesting sites and waters are currently protected under Ecuadorian National Park regulations, the potential impacts from these activities are more evident in MNP (Peña-Mosquera 2010). Including MNP populations in the Galápagos MU would allow for rapid implementation of conservation strategies for coastal populations of green turtles

using the better consolidated conservation scope applied to green turtles in the iconic Galápagos Islands. Another possibility is to extend the current protection of the EP Tropical Marine Corridor to incorporate MNP within this axis of international conservation efforts, a suggestion that stems from the important connectivity shown between MNP, GPS, and Gorgona in Colombia (Amorocho et al. 2012), which should encourage cooperative national and international efforts to reduce anthropogenic effects primarily in MNP. This habitat is valuable regionally not only for green turtles but for multiple marine turtle species, including the Critically Endangered hawksbill. More broadly, and since a significant portion of the turtles at foraging sites in Ecuadorian waters appear to be of WP origin, our study stresses the importance of the conservation value for this region by extending conservation attention across the Pacific, reaching the opposite side of the ocean basin. Finally, and given that the origin of many orphan foraging individuals in the region remains unresolved, we recommend exploration of potential rookery sites north and south of MNP to improve conservation efforts for green turtles along the EP.

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