American Genetic Association

Journal of Heredity, 2015, 448–458
doi:10.1093/jhered/esv050
Symposium Article

OXFORD

Symposium Article

Conservation Genetics of the Scalloped Hammerhead Shark in the Pacific Coast of Colombia

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Data deposited at Dryad: http://dx.doi.org/doi:10.5061/dryad.kq685

Received September 2, 2014; First decision October 6, 2014; Accepted June 26, 2015.

Corresponding editor: Antonio Solé-Cava

Abstract

Previous investigations of the population genetics of the scalloped hammerhead sharks (*Sphyrna lewini*) in the Eastern Tropical Pacific have lacked information about nursery areas. Such areas are key to promoting conservation initiatives that can protect young sharks from threats such as overfishing. Here, we investigated the genetic diversity, phylogeography, and connectivity of *S. lewini* found in 3 areas of Colombia's Pacific coast: around Malpelo Island and in 2 National Natural Parks on the Colombian Pacific mainland (Sanquianga and Ensenada de Utría). We analyzed mtDNA control region (CR) sequences and genotyped 15 microsatellite loci in 137 samples of adults and juveniles. The mtDNA analyses showed haplotypes shared between the Colombian Pacific individuals sampled in this investigation and other areas in the Eastern Tropical Pacific, the Indo-Pacific, and with sequences previously reported in Colombia (Buenaventura Port), as well as 4 unique haplotypes. Population assignment and paternity analyses detected 3 parent–offspring pairs between Malpelo and Sanquianga and 1 between Malpelo and Utría. These results indicate high genetic connectivity between Malpelo Island and the Colombian Pacific coast, suggesting that these 2 areas are nurseries for *S. lewini*. This is, to our knowledge, the first evidence of nursery areas identified for the scalloped hammerhead shark anywhere in the world. Additional conservation planning may be required to protect these nursery habitats of this endangered shark species.

Resumen

Investigación previa sobre genética poblacional del tiburón martillo (*Sphyrna lewini*) en el Pacífico Este Tropical no ha incluido información sobre sus zonas de cría. Estas zonas son claves para promover iniciativas de conservación que protejan tiburones juveniles de amenazas como

sobreexplotación. En este trabajo investigamos la diversidad genética, filogeografía y conectividad *S. lewini* encontrados en tres areas del Pacífico Colombiano: alrededor de la Isla Oceánica de Malpelo y en dos Parques Nacionales Naturales (PNN) en la zona continental (Sanquianga y Utría). Analizamos sequencias de la region control del ADN mitocondrial y genotipificamos 15 loci microsatélites para 137 muestras de tiburones adultos y juveniles. Los análisis del ADN mitochondrial identificaron haplotipos compartidos entre individuos muestreados en el Pacífico Colombiano e individuos muestreados previamente en otras zonas del Pacífico Este Tropical, el Indopacífico y con secuencias previamente reportadas en Colombia (desembarcos en el Puerto de Buenaventura). Se identificaron también cuatro haplotipos únicos para esta población. Los análisis de asignamiento y pruebas de paternidad detectaron tres parejas de parental-cría entre Malpelo y Sanquianga y una pareja parental-cría entre Malpelo y Utría. Estos resultados sugieren alta conectividad genética entre los grupos de tiburones martillos alrededor de Isla Malpelo con zonas continentales, sugiriendo que estas areas son zona de cría *S. lewini*. Este sería la primera evidencia de zonas de cría identificadas alrededor del mundo para esta especie de tiburón martillo. Se requiere planes de conservación con énfasis en la protección de estas zonas de cría para esta especie amenazada de tiburón martillo.

Subject areas: Conservation genetics and biodiversity; Reproductive strategies and kinship analysis
Key words: genetic connectivity, Malpelo Island, shark nurseries, *Sphyrna lewini*

Marine top predators around the world have been exposed to major threats since fishing fleets rapidly expanded in the open ocean, leading to immediate conservation concerns (Baum et al. 2003). Sharks are affected by commercial and by-catch fishing pressures (Baum et al. 2003; Heithous et al. 2008; Lucifora et al. 2011; Mejía-Falla and Navia 2011), and appropriate conservation and management plans are critical, due to their ecological importance, providing significant top-down control over other marine species (Nance et al. 2009). However, the successful development and implementation of such plans has been hampered by the lack of knowledge on the population status of most shark species (Baum et al. 2003).

The scalloped hammerhead shark, *Sphyrna lewini*, is a large predator distributed across tropical and warm-temperate waters, with a coastal-pelagic life history (Daly-Engel et al. 2012). Despite its relatively high fecundity (12–30 pups annually) (White et al. 2008), *S. lewini* has low resilience to exploitation, due to its late sexual maturity (males mature at 10 years and females at 15), and long generation times (around 30 years) (Ovenden et al. 2011). Adult sharks from both sexes are extremely mobile and are often found in the open ocean (Kohler and Turner 2001; Bessudo et al. 2011) where they reproduce annually (Hazin et al. 2001). Females migrate to shallow coastal waters geographically distant from adult feeding grounds, where highly developed pups are born (Holland et al. 1993; Daly-Engel et al. 2012), and remain seasonally resident for 3–5 years. These nursery areas are becoming an essential component of conservation and management plans for shark species (Kinney and Simpfendorfer 2009).

Although not all shark species use nursery areas (Heupel et al. 2007), nurseries have been reported for several species of the family Sphyrnidae (Simpfendorfer and Milward 1993). To date, little is known about the practical value of nursery areas for the recovery of shark populations being harvested around the world (Kinney and Simpfendorfer 2009) and few studies have focused on researching biological aspects of these continental nurseries (Keeney et al. 2003; Chapman et al. 2009). Given the increasing rates of shark population declines, identifying nursery areas has become critical, and conservation and management plans need to be implemented for these particular areas (Heupel et al. 2007). The Colombian National Action Plan for the Conservation and Management of Sharks and Rays of Colombia (Caldas et al. 2010) has, as one of its short-term goals, the identification of shark nursery areas in Colombia, since no information regarding nursery areas is currently available for managers and decision makers in the country.

Population genetic studies have played a key role in defining strategies for fisheries management (Carvalho and Hauser 1994; Castillo-Olguín et al. 2012) and have become valuable for monitoring the effects of exploitation (Bowen et al. 2005; Ovenden et al. 2011). For example, Keeney et al (2005) used mtDNA CR sequences and microsatellite loci to evaluate female vs. male mediated gene flow in the genetic structure of neonates and young-of-the-year blacktip sharks (*Carcharhinus limbatus*) over continental waters of the northwestern Atlantic, Gulf of Mexico, and Caribbean Sea.

Although population structure of *Sphyrna lewini* has been widely investigated on a global and on a regional scale (Quattro et al. 2006; Nance et al. 2011; Daly-Engel et al. 2012), little is known about the genetic connectivity of oceanic adult hammerhead sharks and the juveniles found in continental shallow water.

In this study, we also used a combination of genetic markers that are biparentally inherited (microsatellites) and strictly maternally inherited (mtDNA). Our aims were to study the overall population stucture; to identify the level of genetic connectivity between Sanquianga National Natural Park (Sanquianga) and Ensenada de Utría National Natural Park (Utría) on the Colombian Pacific mainland with the oceanic Malpelo Island (Malpelo), to test if these 2 mainland locations could be considered nursery areas for *S. lewini* in the Colombian Pacific and to provide information for management strategies for this species at the national and regional levels.

Methods

Sample Collection

A total of 137 scalloped hammerhead (*S. lewini*) specimens were sampled from 3 areas along the Pacific coast of Colombia: Malpelo Island

(*N* = 22), Sanquianga (*N* =78), and Ensenada de Utría (*N* = 37). Adult sharks total length ([TL) ≥ 1.5 m] were sampled in Malpelo, while juvenile sharks (TL = 30–50 cm) were sampled in Sanquianga and Utría (Figure 1). For mtDNA analyses, we included additional samples collected between 2009 and 2011 in the port of Buenaventura (*N* = 21) Colombian Pacific (Figure 1). Fin and muscle tissue were preserved in 95% ethanol and stored at room temperature .

Specimens were obtained by a combination of commercial fishery sampling and use of a specially designed biopsy dart during scientific cruises (Bessudo, personal communication). Permits to conduct this research were granted by the Ministerio de Ambiente-Colombia (Contrato de Acceso a Recursos Genéticos para Investigación Científica sin interés commercial No. 093 del 13 de Marzo de 2014).

DNA Extraction, mtDNA Amplification, and Sequencing

Total DNA was extracted from 25 mg of tissue using Phenol: Chloroform: Isoamyl Alcohol (25:24:1, v/v) extraction and ethanol precipitation of DNA adapted from Ausubel et al. (1995). We amplified a 1200 bp fragment of the complete mitochondrial CR from all samples. Reactions were carried out in 25 µL volumes containing 1 µL of genomic total DNA, 1× PCR buffer (QIAGEN Inc.), 200 µM of dNTPs mix (Bioline; Randolph, MA), 3 pmol of each of the primers: Pro-L (5′-AGGGRAAGGAGGGTCAAACT-3′) and 12S rRNA (5′-AAGGCTAGGACCAAACCT-3′), and 1.25 units of HotStar Taq™ DNA Polymerase (QIAGEN Inc.). PCR was performed in a C1000 Touch™ (BioRad, Thermal Cycler with 96-Well Fast Reaction) for 39 cycles of 1 min at 95 °C, 1 min at 61.4 °C, and 2 min at 72 °C, followed by a final extension step of 72 °C for 10 min. Successfully amplified PCR products were purified using Exo-SAP (Thermo Scientific) and sequenced on an ABI 3500 at Universidad de los Andes, Bogotá, Colombia.

Microsatellite Genotyping

All samples were genotyped for 15 previously described microsatellite loci, including 12 species-specific markers isolated by Nance et al. (2009) and 3 non–species-specific markers described by Keeney and Heist (2003). Forward primers were labeled with 6-FAM, VIC, NED, and PET proprietary dyes (Applied Biosystems, Foster City, CA) (Supplementary Table 1 online) . The 15-plex microsatellite PCR reactions were carried out in 20 µL volumes containing: 1.8 mM of $MgCl_2$, 2 Units of AmpliTaq Gold® (Life Technologies, Inc), 200 µM of dNTPs mix (Bioline), 1× PCR Gold Buffer® (Life Technologies, Inc), 0.2× primer mix (with a final primer concentration varying from 0.1, 0.2, or 0.4 µM), Buffer pre-CESII of Ralser et al. 2006 (0.54 M of betaíne, 1.34 mM of DTT, 1.34% of DMSO, and 11 µg/mL of BSA), and 2 µL of genomic total DNA. PCR was performed in a C1000 Touch™ (BioRad, Thermal Cycler with 96-Well Fast Reaction) and consisted of an initial denaturation at 95 °C for 11 min, followed by 30 cycles of 20 s at 94 °C, 2 min at 60 °C, and 1 min at 72 °C, followed by a final extension at 60 °C for 60 min. PCR products were resolved with an ABI 3500 genetic analyzer using LIZ 600 size standard at Universidad de los Andes in Bogota, Colombia and visualized using ABI PRISM GENEMAPPER ID-X Software (Life Technologies, Inc) and GeneMarker software® (Softgenetics LLC, 2010).

Data Analyses

mtDNA Control Region

CR sequences were edited and aligned manually using the software Geneious 5.3 (BioMatters). We determined haplotypes and the number of variable sites using MacClade v3.08 (Maddison and Madisson 2000). For finer-scale *S. lewini* stock delineation, we compared our data with sequences published in DNA databases from 4 biogeographical regions around the world where *S. lewini* is distributed: the Eastern Tropical Pacific (ETP) (Duncan et al. 2006; Nance et al. 2011); the Indo-Pacific Ocean; the Indian Ocean; and the Atlantic Ocean (Duncan et al. 2006; Chapman et al. 2009). Also included in our comparisons were smaller datasets from Belize, Brazil, and the Hong Kong market (Chapman et al. 2009). A haplotype network was constructed using the statistical parsimony methodology as implemented in the software TCS Vs. 1.21 (Clement et al. 2000) (Figure 2).

This method estimates an unrooted tree, providing a 95% plausible set for all sequence type linkages within the tree and considering gaps as a fifth character state. Population structure analyses as well as haplotype (h) and nucleotide (π) diversity calculations were performed in the program Arlequin (Excoffier et al. 2005) and restricted

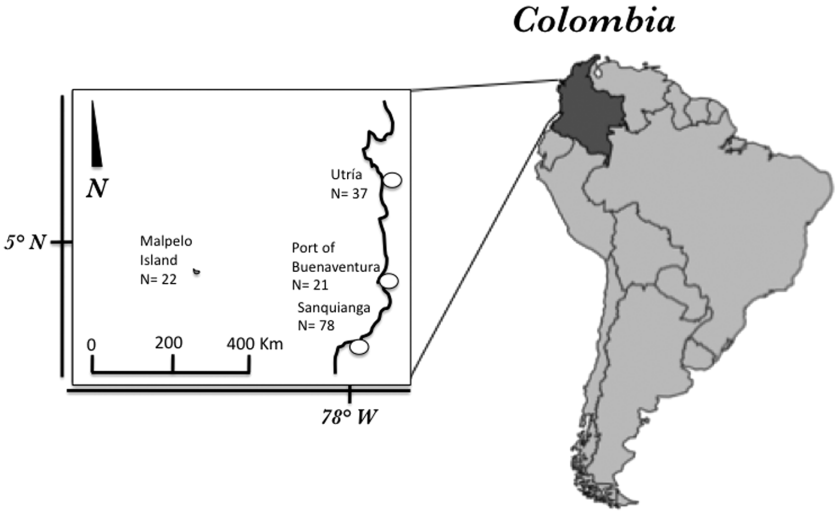

Figure 1. Sampling locations for scalloped hammerhead sharks in Pacific coast of Colombia: Malpelo Fauna and Flora Sanctuary (Malpelo Island), Sanquianga National Natural Park (Sanquianga), Ensenada de Utría National Natural Park (Utria), and Port of Buenaventura.

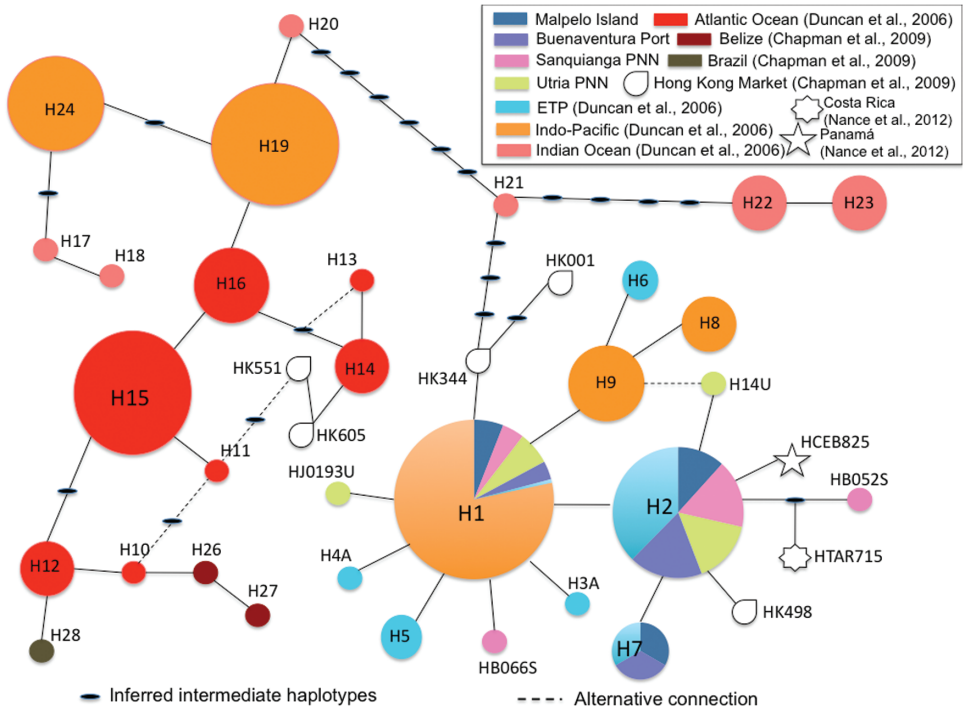

Figure 2. Parsimony network of mtDNA CR haplotypes for scalloped hammerhead sharks.

to 549 bp of the CR. Genetic differences among population units established *a priori* based on geographic locations from previous studies (ETP, Indo-Pacific, Indian Ocean, and Atlantic Ocean) were quantified by an analysis of molecular variance (AMOVA) as implemented in Arlequin (Excoffier et al. 2005) based on conventional *FST* and Φ*ST* statistics, using 10 000 random permutations.

Microsatellite Loci

All loci were tested for presence of null alleles using the software Micro-Checker 2.2.3 – Microsatellite Data Checking Software (van Oosterhout et al. 2004). Nuclear genetic diversity was evaluated by determining the total number of alleles (NA) and the average number of alleles per locus. Expected and heterozygosities (HE and HO), Hardy–Weinberg equilibrium, and population differentiation tests were conducted in GENEPOP® v.4.2 (Raymond and Rousset 1995) and ARLEQUIN SUITE® v. 3.5 (Excoffier and Lischer 2010). F_{IS} was calculated in order to evaluate levels of inbreeding (Weir and Cockerham 1984). This was done for the complete Colombian Pacific population sample (Malpelo, Sanquianga, and Utría) and for each sampling location independently.

In order to reconstruct genealogies, 2 different algorithms were chosen for pedigree analyses. In order to recover related groups from the obtained genotypes, the algorithms were combined in the Pairwise Relatedness option of the software in GenAlEx 6.5® (Peakall and Smouse 2006, 2012). The calculations for several pairwise relatedness estimators were provided by GenAlEx: 1) Ritland (1996), 2) Lynch and Ritland (1999), and 3) the estimator of Queller and Goodnight (1989). The purpose of genetic marker based relatedness estimators is to calculate the fraction of alleles shared among individuals that are identical by descent. Relatedness is a continuous estimate of the overall Identity by Descent (IBD) between individuals. It is similar to but not the same as relationship categories, such as parent-offspring, full sibs, half sibs and so on. which have discrete relatedness estimates of 0.5, <0.5, and 0.25, respectively (See Supplementary Figure 4 online). As an additional alternative method, we used the more conservative software ML-RELATE® (Kalinowski et al. 2006). This software was very useful in discriminating among 4 common genealogical relationships: 1-individuals that are not biologically related (U), 2-half siblings (HS), 3-full siblings (FS), and 4-parent/offspring relationship (POP) through the distribution of pairwise relatedness (*r*) estimates for each of the 4 simulated relationship categories. The sampling variance was calculated as the standard deviation of the mean *r* estimate for each simulation category separately. Comparing the mean tested the bias among estimator *r* and the expected values *r* (U 0.0; HS 0.25; FS and POP 0.5) obtained with a 95% confidence interval (Blouin 2003). This method was selected as ML estimates seems to be more precise in defining relationships among and between siblings (Milligan 2003) .

Population structure was also evaluated with multivariate analyses of principal component (PCoA), factorial correspondence analysis (FCA) using the software GENETIX® v.4.02.2 (Belkhir et al. 2004), and GENEALEX® v. 6.5. (Peakall and Smouse 2006, 2012). In order to estimate the possible number of gene pools and admixture, we used the software STRUCTURE® v. 2.3 (Pritchard et al. 2000). This software is based on a Bayesian algorithm with a grouping method that uses Markov Chain Monte Carlo (MCMC) to estimate the most likely number of gene pools (*K*) in the sample (length burn-in period: 200 000; MCMC: 50 000), and also evaluates different *K* values using population ancestry models.

Data Archiving

In fulfillment of data archiving guidelines (Baker 2013), new haplotypes identified in this study were submitted to Genebank as accession numbers KM922595 to KM922595, and genotypes generated were submitted to DRYAD.

Results

MtDNA Control Region

Sixty-three sequences were analyzed from samples collected in Malpelo (n = 18), Sanquianga (n = 22), and Utría (n = 23). Twenty-one sequences previously obtained from Port of Buenaventura samples (data not published) were included for further comparisons. Among these 84 sequences (Supplementary Table 2 online), 7 haplotypes were defined by 50 variable sites. Of these, 2 (H1 and H2) were shared among all Colombian Pacific locations as well as with the ETP. One of these (H1) was also shared with the Indo-Pacific and appears to be the most ancestral haplotype in the Pacific Region (Figure 2). One additional haplotype, identified in samples from Malpelo and Buenaventura Port (H7), was also shared with the ETP. Four haplotypes were new and unique to the sampling location: 2 identified in samples from Sanquianga and 2 identified in samples from Utría. In the haplotype network (Figure 2), there was a clear separation among haplotypes found in each Ocean basin. Samples identified in Colombian Pacific samples were grouped with haplotypes from the ETP and the Indo-Pacific, clearly separated from haplotypes from the Indian Ocean and the Atlantic Ocean.

There was no significant differentiation of mtDNA in the Colombian Pacific (Malpelo, Sanquianga and Utría) suggesting these all belong to one population unit (Table 1 and Supplementary Table 3 online). There was, however, significant population differentiation between Colombian Pacific locations and the Indo-Pacific, Indian and Atlantic Oceans (Table 1). Some differentiation was detected between Colombian Pacific locations and the rest of the ETP (except for Buenaventura Port) at the mtDNA level, possibly due to the presence of the 4 new and unique haplotypes only found in samples from the Colombian Pacific.

Genetic diversity both at the nucleotide and haplotype level was relatively low for Colombian Pacific locations when compared to the Indo-Pacific, Indian Ocean, and the Atlantic Ocean but was higher than diversity levels previously found in other areas of the ETP (Table 3 and Supplementary Table 3 online).

Microsatellite Loci

One hundred and eleven samples were successfully genotyped for 15 loci from 3 regions of the Colombian Pacific region (Malpelo, Sanquianga, and Utría). All loci analyzed were polymorphic, with an average allele number per loci between 8.356 ± 0.6296. Expected heterozygosity was high (HE = 0.653 ± 0.026) relatively to the observed heterozygosity (HO = 0.559 ± 0.031). An excess of homozygosity was detected in 6 out of the 15 loci (SLE089, CLI100, SLE071, SLE013, CLI12, and SLE077). Deviation from Hardy–Weinberg equilibrium was detected at these loci ($P < 0.00001$) even after a regression or false discovery rate (FDR) correction was applied. No loci showed evidence for linkage disequilibrium. MICRO-CHECKER® analyses revealed 8 loci with evidence of null alleles. However, no systematic deviation was observed at these loci, and they were included in further analyses (Table 2). Heterozygous deficiency was detected in Sanquianga in comparison with samples from Malpelo or Utría. This was also reflected in the inbreeding coefficient (F_{IS}) calculated for Sanquianga (Table 2).

Population structure among sites in the Colombian Pacific was tested in an AMOVA analyses. No significant population differentiation was found among the 3 sites (overall F_{ST} = 0.00499; $P > 0.05$; Table 3).

In the FCA most individuals were grouped in a central axis and only 9 samples were grouped outside it, possibly due to the presence of unique alleles in low frequencies in such samples. The PCoA revealed a similar trend, obtaining one main group of individuals while a small number of samples were placed in the extremes, away from the central group (Supplementary Figures 1 and 2 online). Structure analyses revealed K = 2 as the most probable number of gene pools identified in the sample according to the probability analysis (Ln Probability of data; Supplementary Figure 4 online). Although the probability of K = 2 was slightly greater than that of K = 1, visual inspection of the individual membership coefficients showed no clear population assignment (i.e. all individuals show mixed ancestry; Supplementary Figure 3 online). When making a biological interpretation of these data, we assume this population is a single genetic pool (K = 1) or at most 2. These results also support the hypothesis of connectivity among the sampled locations in this study (Supplementary Figures 3 and 4 online).

Results from GenAlEx 6.5® pairwise relatedness analysis revealed that 24.6% of all samples analyzed had a probable HS relationship ($r \geq 0.25$) and only 4.33% of all samples analyzed could belong to a FS pair (r = 0.4–0.50). Four sample pairs had an $r \geq 0.50$, suggesting a POP relationship. Genotypes of these pairs were checked by eye in order to confirm the possibility of such relationship. After this check, it was concluded that in fact these pairs could have a POP relationship, because these individuals share at least one allele in each of the 15 loci analyzed (Table 4).

Relatedness was also tested in ML-RELATE®. In this analysis, genotyping error rate was established as 5%. Random mating was the standard used in the relatedness analysis. Most samples out of 111 were identified as U (90.94% or 4672 pairs), while a much smaller number of individuals belonged to a HS pair or to a FS pair (8.97% or 461pairs). Only 0.07%, or 4 pairs of all pairs, showed a POP relationship, confirming the results obtained in GenAlEx 6.5® (Table 5). In this Table, POP and FS results are shown, since these were the relationships that we intended to test *a priori*, in order to determine if there was indeed evidence of connectivity. When the software found 2 equal probabilities for POP and FS, the most conservative result was chosen, since in order to assume a POP relationship, the individuals must share at least one allele at each locus (probability of heritability under Mendel laws $k_0 = 0$, $k_1 = 1$, and $k_2 = 0$). This was reviewed by eye and if this was not the case, then a FS relationship was deemed more likely (probability of heritability under Mendel laws $k_0 = 0.25$, $k_1 = 0.5$, and $k_2 = 0.75$).

Discussion

Previous studies have investigated the global phylogeography and population structure of *S. lewini* (Duncan et al. 2006). These studies provided important initial information regarding the levels of genetic diversity and population differentiation of this species in different ocean basins. Also, Nance et al. (2011) provided important initial information about the population structure of this species in the Eastern Tropical Pacific. However, in these studies, no samples from the Colombian Pacific were included. Also, to this date, no information is available regarding identified nursery areas for *S. lewini* in the world. The aim of this research was to investigate the population structure of *S. lewini* in the Colombian Pacific, as well as to test connectivity between oceanic areas were adult sharks are found (i.e. Malpelo) and coastal areas (Utría and Sanquianga) where young animals have been identified and where these individuals are affected by fishing activities.

Table 1. Pairwise F_{ST} (above diagonal) and Φ_{ST} (below diagonal) values for mtDNA CR among 8 sampling locations, worldwide, of scalloped hammerhead sharks

		Colombian Pacific							
	F_{ST} / Φ_{ST}	Malpelo Island	Buenaventura (port)	Sanquianga	Utria	ETP	Indo-Pacific	Indian Ocean	Atlantic Ocean
COLOMBIAN PACIFIC	Malpelo Island (n = 18)	h = 0.58 ± 0.06 π = 0.11 ± 0.001	0.0145	−0.0108	−0.0426	**0.1852**	**0.1944**	**0.2933**	**0.4046**
	Buenaventura (Port) (n = 21)	*−0.0076*	h = 0.52 ± 0.1 π = 0.11 ± 0.001	−0.0358	0.0016	0.0302	**0.3289**	**0.3355**	**0.4310**
	Saquianga (n = 22)	−0.0274	−0.02	h = 0.60 ± 0.09 π = 0.15 ± 0.001	−0.2075	**0.0654**	**0.2806**	**0.2889**	**0.3984**
	Utria (n = 23)	−0.0406	0.0010	−0.0270	h = 0.60 ± 0.06 π = 0.12 ± 0.001	**0.1487**	**0.2182**	**0.2902**	**0.3987**
	ETP (n = 38)	0.0383	−0.0162	0.0102	0.0376	h = 0.42 ± 0.1 π = 0.16 ± 0.001	**0.4460**	**0.4242**	**0.4848**
	Indo-Pacific (n = 183)	**0.2137**	**0.2412**	**0.2270**	**0.2224**	**0.2713**	h = 0.61 ± 0.03 π = 0.91 ± 0.004	**0.3038**	**0.3904**
	Indian Ocean (n = 10)	**0.7232**	**0.7440**	**0.7316**	**0.7460**	**0.7845**	**0.4390**	h = 0.86 ± 0.08 π = 1.27 ± 0.007	**0.2967**
	Atlantic Ocean (n = 43)	**0.9124**	**0.9136**	**0.9100**	**0.9140**	**0.9102**	**0.5900**	**0.7624**	h = 0.60 ± 0.07 π = 0.22 ± 0.001

Probability values based on 10 000 permutations shown in italics.
Significant values ($P < 0.05$) are given in bold.
Haplotype (h) and nucleotide diversities (π)% ± standard deviation are shown on diagonal for each sampling location.

Table 2. Allelic variation of 15 microsatellite loci in 3 samples of scalloped hammerhead shark

Sample		Locus															Average across loci
		Cli-12	Cli-100	Cli-111	SLE013	SLE025	SLE027	SLE028	SLE038	SLE053	SLE054	SLE071	SLE077	SLE081	SLE086	SLE089	
Malpelo	*n*	19	19	18	18	19	19	19	19	19	19	18	19	19	16	17	18.467
	a	7	6	5	2	10	7	7	7	9	4	6	11	8	5	7	6.733
	as	209–287	197–244	166–176	267–285	303–385	421–461	226–260	422–492	398–458	187–219	252–282	224–334	392–422	368–388	181–201	
	He	0.677	0.753	0.38	0.475	0.706	0.535	0.715	0.767	0.738	0.354	0.489	0.794	0.794	0.738	0.834	0.650
	Ho	**0.316**	0.684	0.444	0.333	0.632	0.526	**0.579**	0.789	0.789	0.316	**0.278**	**0.474**	0.842	0.75	0.647	0.560
	HW	**0**	0.273	0.999	0.205	0.153	0.992	**0.002**	0.879	0.57	0.892	**0.009**	**0**	0.486	0.019	0.123	0.373
Utría	*n*	32	33	29	31	33	33	33	33	33	33	33	33	33	28	31	32.067
	a	13	8	4	3	15	9	9	7	8	5	4	17	9	5	7	8.200
	as	209–287	197–244	166–176	267–285	303–385	421–461	226–260	422–492	398–458	187–219	252–282	224–334	392–422	368–388	181–201	
	He	0.789	0.761	0.299	0.005	0.749	0.525	0.751	0.793	0.726	0.385	0.421	0.887	0.759	0.717	0.772	0.623
	Ho	**0.438**	0.788	0.345	0.129	0.697	0.606	**0.697**	0.848	0.606	0.455	**0.212**	0.813	0.727	0.786	**0.548**	0.580
	HW	**0**	0.918	0.974	0.742	1	1	**0.004**	0.725	0.871	0.985	**0**	0.028	0.491	0.254	**0**	0.533
Sanquianga	*n*	57	57	55	57	58	59	58	59	59	59	59	59	59	57	59	58.067
	a	15	9	4	6	19	9	12	10	13	6	5	22	8	5	9	10.133
	as	209–287	197–244	166–176	267–285	303–385	421–461	226–260	422–492	398–458	187–219	252–282	224–334	392–422	368–388	181–201	
	He	0.803	0.768	0.223	0.465	0.795	0.629	0.772	0.793	0.769	0.405	0.325	0.844	0.718	0.673	0.829	0.654
	Ho	**0.404**	**0.649**	0.245	**0.255**	0.638	0.576	**0.776**	0.814	**0.712**	0.322	**0.102**	**0.61**	0.746	0.632	**0.576**	0.537
	HW	**0**	**0**	0.984	**0**	0.198	0.702	**0**	0.842	**0.011**	0.746	**0**	**0**	0.368	0.953	**0**	0.320

P values corrected with FDR (*q* values) are shown in bold.

Significant probabilities of HW deviations and heterozygosity deficit after correction (initial $\alpha = 0.05/x = 0.011$) are shown in bold.

a, number of alleles; as, allele size range; He, expected heterozygosity; Ho, observed heterozygosity; HW, probability of concordance with Hardy–Weinberg expectations.

Table 3. Analysis of molecular variance and pairwise population differentiation from microsatellites for 3 sampling locations of scalloped hammerhead shark using F_{ST} (above diagonal) and standardized R^{ST} (below the diagonal)

Populations	Malpelo Island	Utría NNP	Sanquianga NNP
Malpelo Island	—	0.00736	0.00514
Utría NNP	0.00899	—	0.00415
Sanquianga NNP	0.00778	0.00743	—

Overall F_{ST} values were calculated at $P = 0.17188$. Overall Φ_{PT} values (a measure of population genetic differentiation for diplod data that is analogous to F_{ST} (Peakall 1995; Maguire et al. 2002)) were calculated at $P = 0.14663$.

Φ_{PT}, a measure of population genetic differentiation for diplod data that is analogous to *Fst* (Peakall et al. 1995; Maguire et al. 2002).

Evidence of Genetic Connectivity Between Malpelo Island and Coastal Areas in the Colombian Pacific Mainland

Results from this study provide some interesting findings regarding the population structure of *S. lewini* in the Colombian Pacific and also provide information that complements previous findings regarding the phylogeography of this species. MtDNA CR analyses provided evidence that samples collected in both coastal and oceanic locations in the Colombian Pacific belong to the same population or stock, as common haplotypes among these areas were identified. Additionally, the Colombian Pacific stock is clearly more similar to the Eastern Tropical Pacific and Indo-Pacific populations and highly differentiated from populations from the Indian and Atlantic Oceans, confirming the findings by Duncan et al. (2006). Interestingly, 2 unique haplotypes were identified in Sanquianga and 2 unique haplotypes were identified in Utría. This may be the result of small sampling sizes in Malpelo Island and other areas of the ETP. These haplotypes may be represented in adults from other areas not sampled yet, for example, Cocos Island or the Galápagos Islands, where adults of this species are commonly found, but where no genetic analyses have been conducted yet.

At the nuclear level, no differentiation was found here between Colombian Pacific localities, coherent with our findings on the mtDNA CR. Daly-Engel et al. (2012) also found genetic connectivity and little genetic differentiation using biparentally inherited markers in global shark populations, suggesting that males were generating gene flow in this species and confirming female philopatry.

Additional evidence supporting connectivity between oceanic and coastal areas in the Colombian Pacific for *S. lewini* comes from the Bayesian assignment analyses. These analyses showed that sampled individuals had an assignment probability of belonging to one or at the most to 2 gene pools ($K = 1$ or $K = 2$). Nevertheless, it is important that further research on this species (*S. lewini*) in the Colombian Pacific region includes a larger number of samples to clarify whether there is really evidence for more than one gene pool. Our results to date suggest a strong genetic link between samples collected from adult individuals in Malpelo and juveniles collected in Utría and Sanquianga. This finding correlates with results from Daly-Engel et al. (2012) where the analysis of multilocus structure suggested genetic similarities between samples collected in the Eastern Tropical Pacific, Gulf of Mexico, South Carolina, and Hawaii, where also only one or at most 2 gene pools were identified in genotyped individuals within each region.

Table 4. Pairs of scalloped hammerhead shark samples that share at least one microsatellite allele at each locus, indicating a probably POP pair

sample	Location	SLE089	CLI100	SLE071	SLE086	SLE081	SLE054	SLE028	SLE013	SLE053	CLI111	CLI12	SLE025	SLE027	SLE077	SLE038
58-M	Malpelo	195	210	272	368	408	201	240	277	452	174	245	337	445	246	458
42-S	Sanquianga	195	210	272	378	408	201	240	277	444	166	269	337	459	246	460
85 -M	Malpelo	193	206	272	368	410	201	240	277	436	174	269	347	445	246	458
24-S	Sanquianga	199	214	272	368	406	201	248	277	456	174	269	347	443	246	458
114 -M	Malpelo	195	214	272	368	406	201	238	279	446	166	269	333	447	246	458
1-S	Sanquianga	199	208	272	368	406	201	238	279	436	174	269	333	445	246	458
41-M	Malpelo	199	208	NA	376	408	201	252	279	446	174	269	347	433	240	452
11-U	Utria	199	210	272	376	410	201	240	279	436	174	269	347	445	244	458

Sample, code of the individual. Location codes as in Figure 1, remaining columns are the 15 microsatellite loci analyzed.

Table 5. Likelihood that pairs of scalloped hammerhead shark samples (Ind1 and Ind2) have a POP or FS relationship (*R*), calculated using the program ML-RELATE

Ind1	Ind2	R	LnL(R)	U	HS	FS	POP
91-M	1-M	FS	−48.41	10.29	–	5.72	5.72
11-U	**41-M**	**POP**	**−64.34**	**1.44**	**2.14**	–	–
22-S	14-S	FS	−58.83	4.93	–	1.4	1.4
42-S	**58-M**	**POP**	**−52.78**	**2.46**	**3.73**	–	–
24-S	**85-M**	**POP**	**−62.86**	**2.43**	**4.32**	–	–
B048-S	48-S	FS	−46.91	10.02	–	4.9	4.9
B043-S	B034-S	FS	−48.9	9.24	–	4.51	4.51
1-S	**114-M**	**POP**	**−57.83**	**2.52**	**2.96**	–	–

LnL(*R*) is the natural logarithm of *R*, the relationship with the highest likelihood. Ind1 and Ind2 = pairs of samples. Values in columns U, HS, FS, and POP are the relative LnL for each relationship compared to the relationship with the highest likelihood shown in column R. Probability values indicating POP relationships are shown in bold.

Evidence for Phylopatric Females and Some Migrating Males

One interesting observation, when analyzing the results of FCA and PCoA analyses, is that although most of the genotypes of individuals collected in the 3 Colombian Pacific locations are grouped into a single cluster, 9 individuals (1 from Malpelo, 3 from Sanquianga, and 5 from Utría) were located away from this central group. These samples correspond to individuals whose genotypes are characterized by having some low-frequency alleles. When the mtDNA CR haplotype was checked from these samples, we found that they shared some of the common haplotypes found in these locations (H1 and H2). A possible explanation to this finding is that there may be some philopatric adult females that are usually found around Malpelo Island that may be mating with migrant males coming from other locations in the Eastern Pacific. This would support the idea of male mediated gene flow found in this species by Daly-Engel et al. (2012) and would explain why in biparentally inherited markers, alleles in low frequency are found in a small number of individuals. Similar results have been found in other shark species, such as the lemon shark (*Negaprion brevirostris*) in Bimini, Bahamas (Feldheim et al. 2014), the black tip shark (*Carcharhinus limbatus*) in the Gulf of Mexico (Keeney et al. 2005), and the blacktip reef shark (*Carcharhinus melanopterus*) in French Polynesia (Mourier and Planes 2013).

Identification of *S. lewini* Nursery Areas in the Pacific Coast of Colombia

In this study, we found 4 samples collected from adults *S. lewini* from Malpelo Island identified as potential parents of juvenile sharks (POP pairs) caught in the bays of Sanquianga and Ensenada de Utría National Natural Parks. In each pair, the juvenile shared at least one allele in each of loci analyzed. Each of these POP pairs had probability values (likelihood) based on allele frequencies exceeding 99.9%. This is a highlight in this study, considering the relatively small sample size of adults sampled in Malpelo that were included in our analyses. There is the possibility of increasing the number of POP or FS pairs identified if increased sample sizes from each location are analyzed in the future. This is, in our view, the strongest evidence of connectivity between Malpelo and these coastal areas, and emphasizes the need to better understand the biology of this species in this geographic location. Several studies have been conducted to identify nursery areas in sharks (Keeney et al. 2005; Mourier and Planes 2013; Feldheim et al. 2014). They are a globally threatened group of marine fishes that often breed in their natal region of origin. Female sharks returning to their exact birthplace to breed ("natal philopatry") has been suggested in a number of shark species (Keeney et al. 2005; Daly-Engel et al. 2012; Mourier and Planes 2013; Feldheim et al. 2014). This is, to our knowledge, the first evidence of nursery areas identified for the scalloped hammerhead shark anywhere in the world.

Conservation Implications of This Study

This is the first study identifying nursery areas for *S. lewini*, and it provides important evidence that should be considered in future management plans for this species in Colombia and in the Eastern Tropical Pacific Region. The Colombian Pacific *S. lewini* population, including oceanic and coastal areas, should be considered a distinct stock and conservation management unit. The fact that 4 POP pairs were identified in this study suggests that the current abundance of this species may be low. For that reason, it is very important to start a formal mark/recapture study for *S. lewini* in Malpelo and the coastal areas. Also, additional steps need to be taken in order to protect these sharks around Malpelo, but particularly in the newly described nursery areas of Sanquianga and Utría, where fisheries are a current threat. This information should be included in the National Shark Conservation Action Plan for Colombia. Similar studies should be conducted on other shark species inhabiting Colombian waters in order to create integrated management and conservation plans for these areas.

Funding

Whitley Fund for Nature Foundation, Fondo Patrimonial Fundacion Malpelo, Fundación Malpelo y Otros Ecosistemas Marinos, Iniciativa Genómica, Universidad Javeriana, and Facultad de Ciencias, Universidad de los Andes, Bogotá, Colombia.

Acknowledgments

The authors would like to thank everybody involved in collecting samples included in this study, particularly staff from Sistema de Parques Nacionales Naturales de Colombia and staff from Fundación Malpelo, as well as the local communities and fishermen. The authors are also grateful with D. Castiblanco for generating control region sequences from samples collected in the Port of Buenaventura, included here for comparative analyses, and with P. Bloor, Coordinator of Grupo de Biodiversidad y Recursos Genéticos, Instituto de Genética, Universidad Nacional de Colombia, for helpful discussions on experimental procedures.

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