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Introgressive hybridisation between two widespread sharks in the east Pacific region

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ABSTRACT

With just a handful of documented cases of hybridisation in cartilaginous fishes, shark hybridisation remains poorly investigated. Small amounts of admixture have been detected between Galapagos (*Carcharhinus galapagensis*) and dusky (*Carcharhinus obscurus*) sharks previously, generating a hypothesis of ongoing hybridisation. We sampled a large number of individuals from areas where the species co-occur (contact zones) across the Pacific Ocean and used both mitochondrial and nuclear-encoded SNPs to examine genetic admixture and introgression between the two species. Using empirical analytical approaches and simulations, we first developed a set of 1873 highly informative SNPs for these two species to evaluate the degree of admixture between them. Overall, results indicate a high discriminatory power of nuclear SNPs (F_{ST} = 0.47, $p < 0.05$) between the two species, unlike mitochondrial DNA (Φ_{ST} = 0.00 $p > 0.05$), which failed to differentiate these species. We identified four hybrid individuals (~1%) and detected bi-directional introgression between *C. galapagensis* and *C. obscurus* in the Gulf of California along the east Pacific coast of the Americas. We emphasize the importance of including a combination of mtDNA and diagnostic nuclear markers to properly assess species identification, detect patterns of hybridisation, and better inform management and conservation of these sharks, especially given the morphological similarities within the genus *Carcharhinus*.

1. Introduction

Natural hybridisation refers to species successfully interbreeding and producing viable "hybrid" offspring (Arnold, 1997; Mayr, 1982). Both hybridisation and introgression, which is the incorporation of genetic material from one species into another following hybridisation (Anderson, 1949; Harrison and Larson, 2014), may be important factors for evolutionary diversification (Mallet, 2005; Meier et al., 2017; Seehausen, 2006, 2004). While hybridisation has been well-documented in terrestrial and freshwater organisms, particularly plants (Hemmer-Hansen et al., 2014; Hobbs and Allen, 2014; Kelley et al., 2016; Mallet, 2005; Pujolar et al., 2014), evidence of this process in cartilaginous fishes remains largely unstudied, mainly due to the lack of discriminatory molecular markers, difficulty of sampling, and extreme morphological similarities within speciose families. Furthermore, elasmobranchs (sharks and rays) have internal fertilization involving mate

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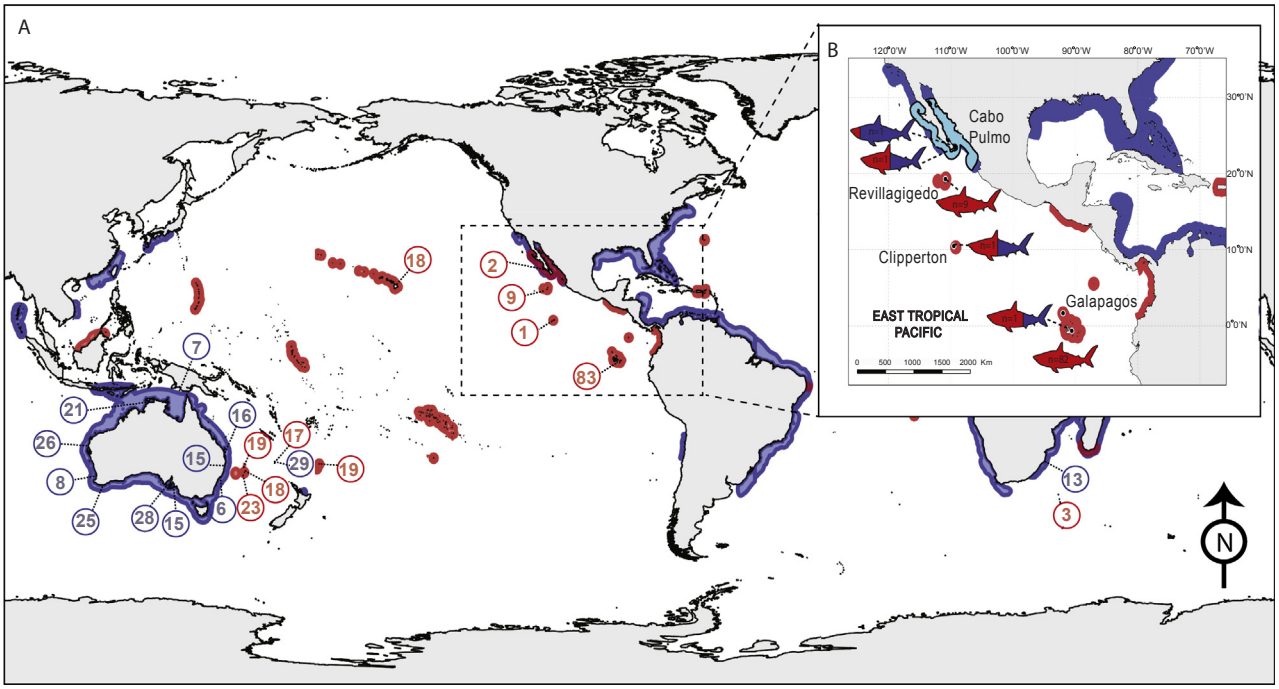

Fig. 1. Map of species distribution, in blue for *Carcharhinus obscurus*, red for *C. galapagensis*, and light blue for areas where both species occur. (A) Sampling locations across the Indo-Pacific, circles indicate the number of *C. galapagensis* and *C. obscurus* (red and blue, respectively) within the study area; (B) East Pacific collection sites and location of hybrids detected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

choice, which adds an additional pre-zygotic barrier to hybridisation within this taxonomic group (Last and Stevens, 2009). Morgan et al. (2012) described the first case of ongoing interspecific hybridisation in sharks between *Carcharhinus tilstoni* and *C. limbatus* (Australian and common blacktip sharks) from eastern Australia. Later, Cruz et al. (2015, 2017) identified and confirmed interspecific hybridisation between the freshwater stingrays *Potamotrygon motoro* and *P. falkneri* in the Parana River (South America) using morphological and genetic (mtDNA, microsatellites and SNPs) data. The same year, Donnellan et al. (2015) confirmed the presence of hybrids between the Eastern and Southern Fiddler Rays (*Trygonorrhina melaleuca* and *T. dumerilii*) in Southern Australia using a combination of mtDNA and SNP loci.

Galapagos (*Carcharhinus galapagensis*, Snodgrass and Heller, 1905) and dusky (*C. obscurus*, Lesueur 1818) sharks are morphologically similar, closely-related species (Garrick, 1982). Precaudal vertebral counts (PVC) and dorsal fin heights are the main morphological characters used to distinguish them (Garrick, 1982). They also differ in habitat preference. The Galapagos shark has a circumglobal distribution in tropical and warm temperate regions, inhabiting mostly isolated oceanic islands, and has a PVC ranging from 103 to 109 (Compagno, 1984; Garrick, 1982; Wetherbee et al., 1996). Despite a few recorded cases of *C. galapagensis* migrating distances of over 2000 km, acoustic telemetry studies from Mexico and Hawaii have identified a preference to remain within 30–50 km of their home range, after being tagged (Lizardi, unpublished data; Kohler et al., 1998; Lowe et al., 2006; Meyer et al., 2010; Papastamatiou et al., 2015). The dusky shark also has a circumglobal tropical to warm temperate distribution, but usually inhabits continental shelves and near-shelf waters, along with a reduced PVC count, ranging from 86 to 97 (Camhi et al., 2008; Compagno, 1984; Garrick, 1982; Last and Stevens, 1994; Rogers et al., 2013). Tagging *C. obscurus* has indicated that long seasonal migrations are common (Bass et al., 1973; Davies and Joubert, 1967; Kohler et al., 1998; Rogers et al., 2013). Importantly, the distributions of Galapagos and dusky sharks only overlap in a few regions, namely Cabo Pulmo National Park, inside the Gulf of California, northeast Pacific ocean (Lizardi, unpublished data; Last and Stevens, 1994); Revillagigedo Islands in the west coast of Mexico, Eastern Pacific (Garrick, 1982); and Norfolk Island off the east coast of Australia, southwest Pacific Ocean (Duffy, 2016). Additionally, occasional reports of Galapagos and dusky sharks have also been recorded along the Ecuadorian mainland coast (Bearez, 2015).

Misidentification between these and other *Carcharhinus* species is common due to morphological similarities (Duffy, 2016; Garrick, 1982; Johnson et al., 2017; Naylor, 1992; Ovenden et al., 2010; Portnoy and Heist, 2012; Tillett et al., 2012), and efforts to resolve their phylogenetic relationships are ongoing. Previous genetic studies using mtDNA have failed to distinguish Galapagos and dusky sharks. For example, Naylor et al. (2012) questioned the validity of Galapagos and dusky sharks after the mitochondrial gene NADH2 (~1044 bp) and failed to distinguish them. However, Corrigan et al. (2017) recently confirmed that *C. galapagensis* and *C. obscurus* are differentiated lineages using a combination of mtDNA and nuclear Single Nucleotide Polymorphisms (SNPs). Thereby demonstrating the relevance of genome-wide data to achieve accurate taxonomic identification in recently diverged systems with limited genomic resources, such as *C. galapagensis* and *C. obscurus*. Corrigan et al. (2017) concluded historic hybridisation had occurred between the two species, resulting in mitochondrial admixture and a small amount of nuclear admixture in Galapagos sharks from the Indo-Pacific and dusky sharks from the Atlantic. However, the authors did not detect signals of ongoing hybridization, most likely due to limited sampling in a single contact zone (Norfolk Island), and recommended increasing sample sizes from sympatric regions.

Under this premise, we predict that genetic exchange may occur between Galapagos and dusky sharks in other contact zones (e.g. along the western Mexican coast), and that it should be possible to detect this by sampling a large number of individuals using genetic markers capable of detecting low levels of admixture, such as genome-wide SNPs. We aimed to explore the possibility of recent hybridization events between *C. obscurus* and *C. galapagensis* by: (1) increasing sampling, particularly at contact zones and (2) developing a panel of diagnostic SNPs to investigate the extent of introgression between *C. galapagensis* and *C. obscurus*.

Table 1
Locations and sample sizes of the Pacific Galapagos shark (*Carcharhinus galapagensis*) and dusky shark (*Carcharhinus obscurus*) populations genotyped for hybridization and introgression assessment using genome-wide SNPs, including four individuals detected as hybrids.

Species	Location	Country	n	Diversity indices		
				$H_{n.b}$ (± SD)	H_o(± SD)	p-value
C. galapagensis	Kermadec Island	New Zealand	19	0.109 (± 0.159)	0.109 (± 0.167)	0.348
	Middleton Reef	East Australia	19	0.107 (± 0.156)	0.108 (± 0.167)	0.349
	Elizabeth Reef	East Australia	18	0.110 (± 0.161)	0.111 (± 0.171)	0.344
	Norfolk Island	East Australia	17	0.106 (± 0.160)	0.106 (± 0.168)	0.322
	Lord Howe Island	East Australia	23	0.109 (± 0.156)	0.109 (± 0.165)	0.312
	Galápagos Islands	Ecuador	82	0.131 (± 0.151)	0.130 (± 0.155)	0.416
	Revillagigedo	Mexico	9	0.137 (± 0.167)	0.140 (± 0.186)	0.530
	Hawaii	U.S.A	18	0.107 (± 0.160)	0.104 (± 0.162)	0.338
	Walters Shoals	South Africa	3	0.104 (± 0.195)	0.101 (± 0.214)	0.236
Total			208			
C. obscurus	Coffs Harbour	New South Wales	15	0.175 (± 0.168)	0.178 (± 0.181)	0.566
	Moreton Bay	Queensland, Australia	16	0.151 (± 0.169)	0.152 (± 0.178)	0.480
	Thirroul	New South Wales	6	0.149 (± 0.188)	0.146 (± 0.205)	0.461
	Indonesia	Indonesia	7	0.160 (± 0.183)	0.161 (± 0.201)	0.537
	Kingscote Jetty	Norfolk Island, Australia	23	0.143 (± 0.169)	0.141 (± 0.174)	0.416
	Northern Territory	North Australia	21	0.147 (± 0.168)	0.144 (± 0.172)	0.418
	South Australia	South Australia	6	0.148 (± 0.186)	0.146 (± 0.202)	0.464
	St. Vincent Gulf	South Australia	9	0.165 (± 0.176)	0.168 (± 0.198)	0.594
	Spencer Gulf	South Australia	28	0.160 (± 0.161)	0.162 (± 0.171)	0.562
	KwaZulu-Natal	South Africa	13	0.138 (± 0.177)	0.132 (± 0.180)	0.386
	Cascade Jetty	Norfolk Island, Australia	6	0.148 (± 0.186)	0.148 (± 0.207)	0.466
	East Cheyne Inlet	Western Australia	15	0.150 (± 0.171)	0.154 (± 0.188)	0.468
	West Cheyne Inlet	Western Australia	10	0.147 (± 0.179)	0.148 (± 0.195)	0.529
	Cape Inscription	Western Australia	26	0.158 (± 0.164)	0.158 (± 0.169)	0.522
	Perth	Western Australia	8	0.155 (± 0.176)	0.149 (± 0.185)	0.552
Total			209			
Hybrids	Cabo Pulmo	Mexico	2	–	–	–
	Clipperton	France	1	–	–	–
	Galápagos Islands	Ecuador	1	–	–	–
Total			**421**			

2. Materials and methods

2.1. Sample collection and DNA extraction

A total of 208 *C. galapagensis* and 209 *C. obscurus* sharks were sampled from across the Indo-Pacific Ocean (Fig. 1A, Table 1). SNPs for all individuals from both species were generated as detailed in Pazmiño et al. (2017) and Junge et al. (2019). MtDNA control region sequences for 22 Galapagos, 22 dusky, and 4 hybrid sharks were sequenced as per Pazmiño et al. (2017) following a modified salting out protocol from Sunnucks and Hales (1996). DNA quality and concentration were determined spectrophotometrically using a NanoDrop 1000 (Thermo Scientific) instrument and agarose gel electrophoresis (0.8% in 1 × TBE containing gel green). Additional *C. obscurus* mtDNA control region sequences were obtained from GenBank (Table 2).

2.2. mtDNA amplification and sequencing

We amplified the mitochondrial control region using a PROMEGA GoTaq Flexi DNA Polymerase kit and control region primers (Light strand ProL2 5′-CTG CCC TTG GCT CCC AAA GC-3′ and heavy strand 282H 5′-AAG GCT AGG ACC AAA CCT-3′) (Keeney et al., 2003; Pardini et al., 2001). PCR reactions were carried out in 25 µl volumes containing 5.0 µl PCR buffer [5X], 1.5 µl $MgCl_2$ [2.5 mM], 0.5 µl [2 mM] deoxynucleotide triphosphates (dNTPs), 0.5 µl each of the Forward and Reverse primers, each at [10 pmol], 0.125 µl Taq DNA Polymerase (5 Units, PROMEGA) and 1 µl of diluted DNA [at a concentration of 10–25 ng/µl]. PCR cycling conditions included an initial denaturation at 95 °C for 2 min, 30 cycles at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1.5 min, and a final extension of 72 °C for 10 min. Pre-stained Biotium Gel-Green agarose gels (1.5%) were used to visualize PCR products. We cleaned PCR products using Sephadex G50 spin columns and sent purified PCR products to Georgia Genomics and Bioinformatics Core Facility (USA) for sequencing.

2.3. mtDNA data analysis

Overall genetic diversity of the CR for each species was assessed as number of haplotypes, haplotype (h), and nucleotide (π) diversity using ARLEQUIN v.3.5.1.2 (Excoffier and Lischer, 2010). Pairwise

Table 2
Sample sizes used to calculate the minimum spanning tree using the Control Region mtDNA (549 bp) for *Carcharhinus galapagensis, C. obscurus* and putative hybrids.

Species	n	Diversity indices		GenBank accession numbers	Source
		h	π		
C. galapagensis	22	0.680 ± 0.066	0.001 ± 0.000	MG241666-241897	Pazmiño et al. (2018)
C. obscurus	22	0.968 ± 0.026	0.007 ± 0.000	HQ853257-853274 MK692872-692875	Benavides et al. (2011) and present study
Putative hybrids	4	0.500 ± 0.265	0.002 ± 0.001	MK692868-692871	Present study
Total	**49**				

differentiation between species (Φ_{ST}) to detect genetic partitioning was calculated in ARLEQUIN after 100,000 permutations. Finally, we drew a Minimum Spanning Tree using POPART (available at: http://popart.otago.ac.nz; Bandelt et al., 1999; Fig. S3).

2.4. Genotyping-by-sequencing and species discrimination analyses

SNP development and genotyping were performed by the DArTSeq Genotyping-by-Sequencing approach developed at Diversity Array Technology Pty Ltd (DArT, Canberra, Australia) as per Sansaloni et al. (2010) and Kilian et al. (2012). DArT SNP calling and quality filtering procedures followed Pazmiño et al. (2017). In order to reduce low-quality and uninformative data (Larson et al., 2014) from the initial data set of 57,341 SNPs, we used the custom *dartqc* pipeline (available at: https://github.com/esteinig/dartQC) to filter SNPs according to: (1) call rate (callrate = 100%); (2) Minor Allele Frequency (MAF > 0.02); and (3) duplicate SNPs (with identical cloneID), keeping the best SNPs based on MAF score only. The output file was manually converted into a GENEPOP format file and transformed into different formats as required using PGDSpider v2.0.6.0 (Lischer and Excoffier, 2012). Our filtered dataset comprised 1873 genome-wide SNPs.

Measures of genetic diversity, including observed (H_o), and unbiased expected (H_{nb}) heterozygosity (corrected for population sample size) were calculated at the intraspecific level using GENETIX v.4.05.2 (Belkhir et al., 2004) and ARLEQUIN v.3.5.1.2 (Excoffier and Lischer, 2010). Pairwise F_{ST} between species was calculated according to θ of Weir and Cockerham (1984) and Nei's minimum distance (Nei, 1978). Following SNP quality checks and filtering we evaluated relatedness among individuals within and between species using STRUCTURE v2.3.4 (Pritchard et al., 2000) and NETVIEW P R package (Neuditschko et al., 2012; Steinig et al., 2016). The first analysis comprised 10 independent runs performed with a burn-in of 100,000 steps, followed by 1,000,000 additional Markov Chain Monte Carlo (MCMC) iterations. An admixture ancestry model was assumed with independent allele frequencies and no population priors. The latter analysis consists of three components: (1) calculation of an Identity-by-Similarity (IBS) distance matrix reconstructed in PLINK, which relies on allele-sharing distance (ASD); (2) a minimum spanning tree reconstruction; and (3) a network construction using nearest neighbor thresholds (k-NN) ranging from 10 to 100 (Purcell et al., 2007; Neuditschko et al., 2012). Individuals that did not either fall within one of the two parental clusters or were of mixed ancestry were removed, and a total of 100 individuals (50 from each species) were then selected as pure parental individuals (q > 99.5%) for further simulations and analysis.

In order to select diagnostic markers from the total set of SNPs, F statistics were calculated for each locus with the PEGAS R Package (Paradis, 2010). Two different subsets of diagnostic unlinked markers were selected based on genetic differentiation (F_{ST}) values of either $F_{ST} > 0.90$ or $F_{ST} > 0.95$. STRUCTURE and NETVIEW P analyses were run with both diagnostic marker data sets (117 SNPs with $F_{ST} > 90$ and 69 SNPs with $F_{ST} > 95$).

2.5. SNP validation and hybrid identification

To test the power of the selected SNPs to assign/classify individuals, we used a simulation approach using HYBRIDLAB v.1.1 (Nielsen et al., 2006). The fifty purest individuals of each species from the initial STRUCTURE run were selected as pure parents to simulate the hybrid classes. Both parental classes, plus eight hybrid class categories were generated with 50 random simulated genotypes each: (1) Pure *C. galapagensis* (Gal), (2) Pure *C. obscurus* (Obs), (3) first generation (F_1) hybrids, (4) second generation (F_2) hybrids, (5) first-generation backcross between Gal and F_1 hybrids (bGal), (6) first-generation backcross between Obs and F_1 hybrids (bObs), (7) second-generation backcross between Gal and bGal, (8) second-generation backcross between Obs and bGal, (9) second-generation backcross between Obs and bObs, and (10) second-generation backcross between Gal and bObs. Simulated data was then reassigned to their most likely hybrid class using two Bayesian assignment methods: First we run STRUCTURE using the same parameters as per empirical data. Then we performed a Bayesian assignment method using NEWHYBRIDS v.1.1 (Anderson and Thompson, 2002) to determine the posterior probability that each individual belongs specifically to one of the ten categories previously simulated. The second assignment method was also applied to pure parental individuals plus the putative hybrids (n = 104). The run used uniform priors for a burn-in of 100,000 sweeps, followed by 1,000,000 MCMC iterations in each analysis. The frequency classes with the respective expected proportions are summarized in Table S1.

3. Results

3.1. Pure parental and hybrid identification based on SNPs

Overall, high genetic differentiation was observed between *C. galapagensis* and *C. obscurus* ($F_{ST} = 0.47$, $p < 0.05$) using nuclear SNPs. Overall genetic diversity in *C. galapagensis* ($H_o = 0.118$; $H_{n.b} = 0.121$) was lower than in *C. obscurus* ($H_o = 0.153$; $H_{n.b} = 0.153$). NETVIEW P network visualization from all individuals based on 1873 SNPs at a maximum number of nearest neighbors (KNN) of 190, clearly showed close relatedness of conspecifics. Four individuals fell outside of the two discrete species clusters and were considered putative hybrids: one from the southern Galápagos Islands (RSN3), two from Cabo Pulmo (MX1, MX2) and one from Clipperton Island (France) off the west coast of Mexico (MX13) (Fig. 1B). Three of these individuals (RSN3, MX1, and MX13) were connected (intermediate) to the two species clusters, but MX2 appeared more related to the *C. obscurus* cluster (Fig. 2A). When all SNP loci were used, MX2 separated from the core *C. obscurus* cluster. A similar pattern was detected by the initial STRUCTURE analysis including all 421 samples, with the same four individuals showing high levels of admixture between the two species (Fig. S1). Additionally, two individuals from Norfolk Island (Cgal_N1321_77 and Cgal_N1321_80) originally labeled as *C. galapagensis,* clustered within the dusky group, and were relabeled as *C. obscurus,* based on this result. After removing the putative hybrids, we selected the individuals with the highest probability of assignment to one or the other cluster, with alternatively fixed alleles. We subsequently defined these individuals as pure parental *C. galapagensis* or *C. obscurus* (Table S2).

3.2. Simulated data

Following the pure parental selection, individual locus F_{ST} values calculated in PEGAS were used to filter and select two discriminant sets of loci at two F_{ST} thresholds: (a) 117 loci with $F_{ST} > 0.90$ and (b) 69 loci with $F_{ST} > 0.95$ (Table S3). These subsets were then used to simulate both pure parental, and eight hybrid classes (including, F_1, F_2, first and second-generation backcrosses in both directions). A total of 500 individuals were simulated for each data set, fifty per hybrid class, to test the power of the selected markers. STRUCTURE runs for the first simulated data set (117 SNPs; $F_{ST} > 90$) showed a clear differentiation among pure parental and all hybrid classes, except for F_1 and F_2 hybrids. While the difference among the other hybrid categories was about 12%, F_1 and F_2 showed a similar proportion of admixture (0.499) and could not be distinguished from one another (Fig. 3A). The second simulated data set (69 SNPs; $F_{ST} > 95$) showed a similar trend. However, the accuracy around *Q* was lower in general when using fewer SNPs, and more variable in later generation backcrosses (Fig. 3B). When testing the power of the first SNP panel (117 SNPs; $F_{ST} > 90$), all simulated individuals corresponding to parental classes (pure *C. galapagensis* and *C. obscurus*), F_1 and F_2 hybrids were correctly assigned by NEWHYBRIDS to their corresponding class. For first and second-generation backcrosses, accuracy ranged from 98 to 99 per cent (Fig. S2A). For the second and smaller data set (69 SNPs; $F_{ST} > 95$),

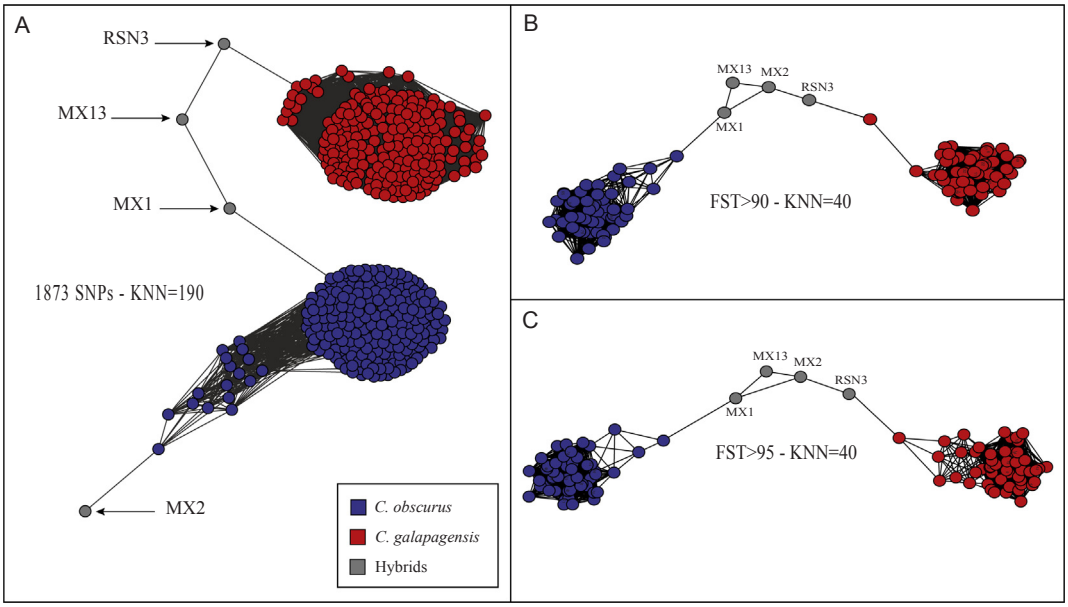

Fig. 2. Network reconstruction with Netview P v.0.4.2.5 to evaluate relatedness among individuals of *C. galapagensis* (red), n = 208 and *C. obscurus* (blue), n = 209, using: (A) all individuals (n = 421) and all filtered SNPs (1873 loci); (B) pure parental individuals (n = 100, 50 per species) and SNPs with F_{ST} > 0.90 (117 loci); and (C) pure parental individuals (n = 100, 50 per species) and SNPs with F_{ST} > 0.95 (69 loci). Hybrids are shown in gray. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

NEWHYBRIDS correctly classified all the individuals from the parental *C. galapagensis* class, F_1, and F_2 hybrid classes. Accuracy for the parental *C. obscurus* class was 99 per cent, and ranged from 93 to 99 for first and second-generation backcrosses (Fig. S2B).

3.3. Empirical data

Two clusters (k = 2) were identified as the best scenario using STRUCTURE HARVESTER. From a total of 104 individuals used for this analysis, 50 were assigned as *C. galapagensis*, 50 as *C. obscurus*, and four were identified as putative hybrid individuals by an initial NETVIEW P analysis, which showed different levels of admixture between the two clusters. The putative hybrids showed similar levels of admixture in both SNP subsets (Fig. 3C–D). Results from NEWHYBRIDS assignment were congruent with those from STRUCTURE (Fig. 3E–F). The same four individuals (RSN3, MX1, MX2, and MX13) were assigned in the NEWHYBRIDS analysis to non-parental classes. In the analysis based on 117 SNPs, hybrids were assigned to three classes: (1) RSN3 was assigned as a second generation backcross between a *C. galapagensis* and a first generation backcross *C. obscurus* with a posterior probability of 0.99; (2) MX2, and MX13 were assigned as F_1 hybrids with posterior probabilities of 1.00 and 0.76 respectively; and (3) MX1 was assigned as a second generation backcross between a *C. obscurus* and a first generation backcross of the same species with a posterior probability of 0.99 (Fig. 3E, Table S4a). In the analysis based on 69 SNPs three hybrid classes were also detected. However, not all the individuals were assigned to the same class as per the previous data set: (1) RSN3 was classified as a second-generation backcross class (bObs × Gal); (2) MX2 was assigned as an F_2 hybrid with posterior probability of 1.00; (3) MX1 and MX13 were assigned to the second-generation backcross between *C. obscurus* and a first-generation backcross of the same species, both with a posterior probability of 0.99 (Fig. 3F, Table S4b). Network visualizations from the two subsets of SNPs (F_{ST} > 90 and F_{ST} > 95) at KNN = 40 showed a similar topology (Fig. 2B–C). The presence of two well-defined clusters, one for each species, is congruent with the STRUCTURE clustering results. All individuals, except the four putative hybrids, were assigned to either the *C. galapagensis* or the *C. obscurus* cluster. Hybrid individuals formed an intermediate cluster linking the two main clusters (Fig. 2B–C).

3.4. mtDNA species determination

Twenty control region haplotypes were detected among 49 individuals: *C. galapagensis* (n = 23), *C. obscurus* (n = 22), and four putative hybrids (based on the above-mentioned SNP analyses) between the two species (Table 2). Overall haplotype and nucleotide diversities ranged from $h = 0.680 \pm 0.066$ and $\pi = 0.001 \pm 0.000$ for *C. galapagensis* to $h = 0.968 \pm 0.026$ and $\pi = 0.007 \pm 0.000$ for *C. obscurus*. A total of 19 polymorphic sites were detected, 13 of which were parsimoniously informative. As expected, *C. galapagensis* and *C. obscurus* were indistinguishable using control region data ($\Phi_{ST} = 0.00p > 0.05$). The mitochondrial control region minimum spanning tree clustered *C. galapagensis* and *C. obscurus* together and a total of three shared haplotypes were observed (H1, H9, and H10; Fig. S3).

4. Discussion

4.1. SNP validation, species determination and hybrid detection

This is the first study to document evidence for contemporary hybridisation between Galapagos (*Carcharhinus galapagensis*) and dusky (*Carcharhinus obscurus*) sharks. We found different levels of admixture between individuals from three distant east Pacific locations (> 1000 km apart): the Galápagos Islands (Ecuador), Cabo Pulmo (Mexico), and Clipperton Island (French DOM-TOM, Eastern Tropical Pacific). We identified the level, frequency, and direction of hybridisation and introgression between these two species by developing and genotyping 1873 genome-wide SNPs. Genome-wide SNPs have been identified as effective markers for addressing cryptic speciation, hybridisation and introgression questions, because of their easily interpretable biallelic nature, low mutation rate, low homoplasy and high power to detect several hybrid classes (Balloux and Goudet, 2002; Cruz et al., 2017; Pujolar et al., 2014). We also highlight the importance of a robust and broad sampling strategy across the Pacific distribution of both species, especially from contact zones, together with

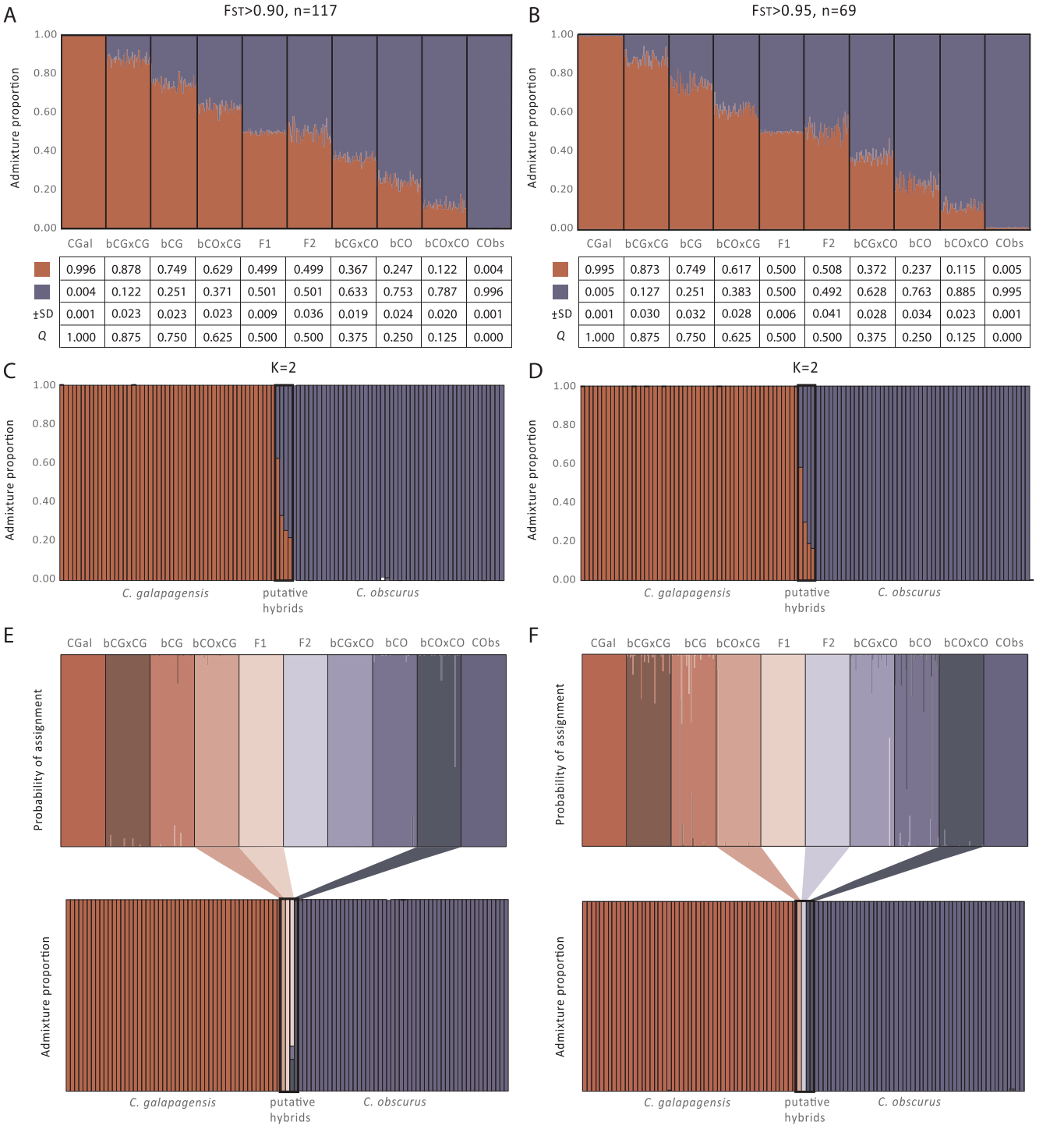

Fig. 3. Bayesian admixture analysis in STRUCTURE of (A) simulated individuals and 117 SNPs with $F_{ST} > 0.90$; (B) simulated individuals and 69 SNPs with $F_{ST} > 0.95$; (C) empirical data and 117 SNPs with K = 2; (D) empirical data and 69 SNPs with K = 2; assignment of putative hybrids and pure parental individuals to the corresponding simulated category using (E) 117 SNPs and (F) 69 SNPs. Simulated categories include: pure *Carcharhinus galapagensis* (Gal), pure *Carcharhinus obscurus* (Obs), F_1 hybrids, F_2 hybrids, first-generation backcross – GalxF_1 (bG), first generation backcross – ObsxF_1 (bO) and second generation backcrosses (bGxG, bOxG, bGxO, bOxO). Observed admixture proportion values and their standard deviation (± SD) and expected proportion values (Q) are included for each simulated category.

comprehensive quality assessment and a data filtering process to accurately define species relationships and detect rare hybridisation and introgression. Overall, our results caution against the unrealistic expectation that single mtDNA markers inherently offer discriminatory power.

While challenging, correct selection of pure parental individuals is crucial to identify unbiased diagnostic markers (Nussberger et al., 2013). We defined 50 individuals (reference individuals) from each parental species based on a STRUCTURE clustering analysis to identify pure parental representatives of both species. Thus, we retained only samples without any evidence of admixture (probability of assignment > 99%) across 1873 loci to define diagnostic SNP panels for each species. Ten categories, including pure parental species, F_1, F_2, first and second-generation backcrosses were successfully simulated with a

posterior probability greater than 0.98. Nussberger et al. (2013) raised concern regarding overestimation of SNP power given the initial selection of pure parental individuals. However, given the high level of SNP differentiation between our two parental shark species we do not expect significant bias in our results. Additionally, selection of loci for diagnostic purposes is a complex matter and must be carefully balanced to retain an appropriate number of loci with discriminatory power. Selection based on high F_{ST} values only, might lead to reduced data sets if the F_{ST} threshold is set too high, which may therefore lead to reduced accuracy. Both subsets of diagnostic unlinked SNPs ($F_{ST} > 0.90$ and $F_{ST} > 0.95$) consistently identified the same four hybrid individuals from different hybrid classes (~1% of the total sample examined). However, the subset of 117 $F_{ST} > 0.90$ diagnostic SNPs with call rate of 1.00, Minor Allele Frequency of 0.02, had better accuracy in identifying hybrids up to the third generation compared to the 69 $F_{ST} > 0.95$ diagnostic SNP markers.

Our findings are consistent with those of Corrigan et al. (2017), who used NADH2 mtDNA and 2152 nuclear SNPs to investigate the relationship between Galapagos and dusky sharks. They suggested historical hybridisation prior to species isolation, and acknowledged a need to obtain a larger number of samples from both species to further evaluate this matter. Our study sampled threefold more samples than Corrigan et al. (2017), with approximately evenly distributed sampling between species, and included three contact zones: (1) From Norfolk Island we sequenced SNPs for 17 Galapagos and 29 dusky sharks, compared to Corrigan et al. (2017), who produced genomic SNP data for one Galapagos and five dusky sharks there; (2) from Cabo Pulmo in the Gulf of California, we included two samples that were originally labeled as dusky shark, but then reassigned as hybrids; and (3) from the Revillagigedos archipelago off the western coast of Mexico we also included six Galapagos shark samples.

Our results identified one hybrid from the southern Galápagos Islands (RNS3), corresponding to a second-generation hybrid backcross between a pure *C. galapagensis* (Gal) and a first-generation *C. obscurus* backcross (Obs × F_1 hybrid). While Galapagos sharks are common in the Galápagos Islands, the closest dusky sharks to this region were reported 1000 km east of the Galapagos Islands, in Ecuadorian continental shelf waters where Galapagos sharks have occasionally been reported (Bearez, 2015). The presence of unreported dusky sharks in the islands is unlikely, given the extensive sampling effort in the archipelago and continuous monitoring by the Galápagos National Park authorities. Both species are more likely to make contact along the continental shelf where they could be reproducing. Mexican hybrid individuals MX1 and MX13, both sampled from the Cabo Pulmo contact zone in the Gulf of California, corresponded to a second-generation *C. obscurus* backcross (F_2 hybrid × Obs) and an F_1 hybrid, respectively. Dusky sharks occur throughout the west coast of Mexico (Garrick, 1982; Musick et al., 2009), which explains the presence of hybrids with more dusky genetic material in Cabo Pulmo. The fourth individual, MX2, is an F_1 hybrid collected from Clipperton Atoll, 965 km west of Mexico, at the edge of the Eastern Pacific Barrier (Snodgrass and Heller, 1905). Despite significant sampling of both Galapagos and dusky sharks at Norfolk Island (n = 17 and n = 29, respectively), we were not able to detect hybridisation in this particular contact zone, probably because both species are common there (Duffy, 2016). On the other hand, differences in abundance between these species have been reported in locations such as the Revillagigedo Islands and Clipperton Atoll, where Galapagos sharks are common while dusky sharks are not (Garrick, 1982). A review of the growing literature on reef fish hybridisation highlights that hybridisation is most prevalent between closely-related species, particularly when one of the species is rare in the contact zone resulting in the lack of conspecific partners (Montanari et al., 2016, 2014). With shark populations declining worldwide, the differential patterns of abundance are likely to increase in areas of co-occurrence, therefore increasing chances of hybridisation. However, extended sampling from locations where we detected hybridisation, and across the west coast of Central and South America is needed to examine more areas of co-occurrence of the two species (e.g. Cocos and Malpelo Islands), and to estimate the frequency of occurrence of hybridisation in these locations. Furthermore, re-classification of both individuals from Norfolk Island (Cgal_N1321_77 and Cgal_N1321_80) could not be confirmed in the absence of PVC data, and is based solely on SNPs.

The presence of an F_1 hybrid at Clipperton Atoll and a second-generation backcross (with more *C. galapagensis* genetic material) at the Galápagos Islands suggests movement of female Galapagos sharks (potentially hybrid mothers) from the primary area of contact (Gulf of California or Revillagigedo) towards the Galápagos Islands using Clipperton Atoll as a stepping-stone. The possibility of these F_1 and second-generation backcross hybrids migrating from the area of contact is low since the hybrid individuals from Clipperton Atoll and the Galápagos Islands were both juveniles and most likely born at these locations, and therefore deduced to be less likely to travel long distances compared to adults (Lizardi, unpublished data; Meyer et al., 2010). Our results suggest hybrids are reproductively viable given the presence of backcrossed hybrids up to the second generation. However, our knowledge of hybrid fitness is limited and further investigation is required to better understand the dynamics of mating in these two closely-related shark species, particularly during hybridisation. Based on our findings, we suggest that both species maintain their habitat and mate preference, evident from the rarity of hybridisation (~1%). Finally, we agree with Morgan et al. (2012) on the importance of considering inter-species hybridisation when using mtDNA for species identification in sharks, as hybrids may be either missed or mis-assigned. Therefore, combining molecular (both nuclear and mitochondrial), morphological and ecological approaches is crucial to elucidate historical and contemporary factors that promote genetic exchange among species.

4.2. Implications for conservation

Understanding the role of hybridisation (if occurring) in commercially important species that are difficult to distinguish, such as dusky and Galapagos sharks, is essential to achieve appropriate species identification, and therefore to monitor catches and produce accurate estimations of population productivity to ensure long-term sustainable fisheries (Simpfendorfer and Dulvy, 2017; Ovenden et al., 2010; Portnoy and Heist, 2012; Tillett et al., 2012). The study of hybridisation and the potential of hybrids to adapt to changing environments and occupy unexploited ecological niches in bony fishes is leading the way towards a better understanding of processes such as adaptation and speciation in the oceans (Montanari et al., 2012, 2016; Hobbs and Allen 2014; DiBattista et al., 2015). Further elasmobranch studies, including the identification of morphological characteristics of hybrid individuals, and their fitness are crucial to understand the evolutionary implications of these hybridisation events.

From a practical point of view, when diagnostic external morphological characters are lacking, or when identifications rely on body parts, the use of non-expensive techniques such as mtDNA sequencing is often used to aid taxonomic identification. However, when dealing with closely related, recently diverged species, as is the case of *C. galapagensis* and *C. obscurus*, the combination of mtDNA and genome-wide diagnostic markers is required to distinguish the species and also allows other biologically relevant patterns of genetic variation to be explored, such as those that arise due to hybridization. Interpreting patterns of mitochondrial genetic variation in isolation could lead to the interpretation that *C. obscurus* and *C. galapagensis* are conspecific. However, by having developed large, multi-marker datasets we now know that these are good species, that they have admixed mtDNA across their range probably due to ancestral hybridization events, that hybridization appears to be ongoing and produces viable offspring and that this seemingly occurs in contact zones where there is uneven abundance of these species such that opportunities for conspecific mating are limited

for one of the species.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.04.013.

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