



Short Communication

Historical introgression drives pervasive mitochondrial admixture between two species of pelagic sharks



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ABSTRACT

We use a genomic sampling of both nuclear and mitochondrial DNA markers to examine a pattern of genetic admixture between *Carcharhinus galapagensis* (Galapagos sharks) and *Carcharhinus obscurus* (dusky sharks), two well-known and closely related sharks that have been recognized as valid species for more than 100 years. We describe widespread mitochondrial-nuclear discordance in which these species are readily distinguishable based on 2152 nuclear single nucleotide polymorphisms from 910 independent autosomal regions, but show pervasive mitochondrial admixture. The species are superficially morphologically cryptic as adults but show marked differences in internal anatomy, as well as niche separation. There was no indication of ongoing hybridization between the species. We conclude that the observed mitochondrial-nuclear discordance is likely due to historical mitochondrial introgression following a range expansion.

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1. Introduction

Carcharhinus galapagensis (Snodgrass and Heller, 1905) and *C. obscurus* (Lesueur, 1818) are closely related shark species that have been recognized as distinct for more than 100 years. While the two species look superficially similar as adults, they have different numbers of pre-caudal vertebrae (Garrick, 1982), as well as marked and consistent differences in onset of maturity (Natanson et al., 1995; Simpfendorfer et al., 2002; Wetherbee et al., 1996). They also occupy different niches: *Carcharhinus galapagensis* prefers shelf and reef habitats, most often occurring at offshore islands and seamounts, while *C. obscurus* are primarily found along continental margins (Ebert et al., 2013). Notwithstanding these differences, preliminary molecular analysis (see Naylor et al., 2012b) based on the mitochondrial DNA NADH2 marker (which is widely used

to identify elasmobranch species) showed that *C. galapagensis* individuals sampled near Hawaii were admixed with *C. obscurus* individuals that were sampled from throughout the Pacific Ocean. While these species are known to co-occur at Norfolk Island, sympatric occurrences are generally uncommon and *C. obscurus* has not been recorded off Hawaii. This precludes taxonomic misidentification as a potential cause for the observed mitochondrial admixture and suggests that either the NADH2 marker is failing to resolve these species because there is mitochondrial introgression between them (or perhaps because of incomplete lineage sorting or some form of selection at the mitochondrial level), or that the two forms are not actually valid species, but are instead ecologically driven morphological variants of the same species.

Accurately inferring taxonomic boundaries is fundamental to our understanding of biodiversity and also has implications for the conservation management of exploited species such as *C. galapagensis* and *C. obscurus*, which are targeted in artisanal, recreational and commercial fisheries. Molecular data can be powerful for discriminating species, but it is well-recognized that inferring

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relationships based on a small number of molecular markers is problematic because of the potential for discordance between gene trees and species trees (Maddison, 1997). In this study we explored the pattern of genetic admixture between *C. galapagensis* and *C. obscurus* using a genomic-scale sampling of both nuclear and mitochondrial DNA markers, sampled from individuals throughout the respective ranges of both species, including their type localities and one location where they occur in sympatry. We used these data to explore the possibilities that these species hybridize or are conspecific.

2. Materials and methods

2.1. Sample collection and sequencing

Muscle samples were collected from 128 individuals nominally identified as *C. galapagensis* (N = 53) or *C. obscurus* (N = 75) based on their external morphology and sampling location (offshore islands/seamounts versus coastal, Fig. 1). Samples were collected from across the distribution range of both species, including sites in the Atlantic Ocean basin and the Indo-Pacific (Fig. 1, Table S1). The type locality for each species was sampled (Galapagos Islands for *C. galapagensis*, east coast U.S.A. for *C. obscurus*), as well as Norfolk Island where these species are known to occur in sympatry (Table S1). Tissue was stored in 95% ethanol prior to DNA extraction using the E.Z.N.A.[®] Tissue DNA Kit (Omega Bio-Tek, Inc., Georgia USA).

Universal primers were used to amplify the mitochondrial NADH2 fragment by Polymerase Chain Reaction (PCR) for all samples prior to purification and Sanger sequencing, following Naylor et al. (2012a).

A subset of samples (N = 11 *C. galapagensis*, N = 15 *C. obscurus*) that were representative of the overall sampling effort (including individuals sampled in sympatry at Norfolk Island and the individual sampled off Senegal which possessed an unusual mitochondrial haplotype, see below) were subjected to targeted DNA hybridization capture for the purpose of collecting nuclear sequence data (Table S1). Following Li et al. (2013), Illumina sequencing libraries (500 bp) were prepared and amplified by PCR prior to two rounds of target gene capture (1077 independent autosomal protein-coding regions). Enriched libraries were pooled and sequenced (paired-end 250 bp reads) on an Illumina MiSeq benchtop sequencer (Illumina, Inc, San Diego, CA).

2.2. Data matrix assembly

NADH2 sequences were aligned using Geneious[®] Pro v.6.1.7 (Biomatters Ltd, Auckland, New Zealand), yielding a nucleotide alignment that was 1044 bp in length.

De novo reference sequence assembly, read mapping, variant calling, and filtering for the nuclear data is detailed in Maisano Delser et al. (2016). Adapters and low-quality reads were removed using cutadapt and FastQC, available in the wrapper script Trim Galore! v0.3.1 (Krueger, 2012). Trimmed sequence reads from several individuals were first used to build a haploid reference sequence for the 1077 target exons and their associated introns. Contigs were assembled de novo using ABySS v1.3.6 (Birol et al., 2009). On-target material was identified by comparing the assembled contigs to a set of single-copy core-orthologs that were found to be conserved across model vertebrates using HaMStR v13.2.3 (Ebersberger et al., 2009). For each exon and intron, the longest contig across all sequenced individuals was retained, which resulted in an 836,916 bp reference sequence including exons plus 5' and 3' introns. Trimmed reads from all individuals were then mapped to the reference sequence using the Burrows-Wheeler

Aligner (BWA, Li and Durbin, 2009). Duplicate read marking was performed with Picard v.1.129 (<http://broadinstitute.github.io/picard/>), local realignment with the Genome Analysis ToolKit (GATK) v.3.3.0 (McKenna et al., 2010) and variant calling with SAMtools v.1.1 (Li, 2011). Raw variants were filtered for strand bias, minimum 6× depth of coverage, triallelic sites, sites with heterozygous calls in more than 80% of samples and missing data (Table S2) using custom Perl (v.5.18.2) and R (v.3.0.2) scripts (Table S3). This resulted in data for 2152 high-quality nuclear SNPs from 910 independent loci (Table S2).

2.3. Data analysis

NETWORK v.5 (Fluxus Technology Ltd.) was used to generate a median-joining network of mitochondrial NADH2 haplotypes, with MP post-processing (substitution weights = 10 and epsilon = 0). In addition, maximum likelihood analysis (GTR + G) was conducted using RAxML v.7.3.0 (Stamatakis et al., 2005) with 1000 rapid bootstrap replicates (Stamatakis et al., 2008). *Carcharhinus longimanus* and *C. perezi* were used as outgroups.

Hierarchical Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992; Weir and Cockerham, 1984; Weir, 1996) was used to quantify interspecific divergence relative to divergence among ocean basins, for both the mitochondrial NADH2 and the nuclear SNP datasets. Four populations were defined: *Carcharhinus galapagensis* from the Atlantic, *C. galapagensis* from Indo-Pacific, *C. obscurus* from the Atlantic and *C. obscurus* from the Indo-Pacific. Two separate AMOVA were conducted in which the four populations were grouped either by species or by ocean basin. Total variance was partitioned into within-populations, among-populations-within-species (or ocean basin) groups and among-species (or ocean basin) groups components.

Principal component analysis (PCA) was performed on the filtered nuclear SNP dataset with the function “prcomp” in the R environment (R Core Team, 2014). The number of clusters present in the data was further investigated using model-based clustering in STRUCTURE v.2.3.4 (Pritchard et al., 2000) with 100,000 iterations (30,000 burn-in). The number of assumed populations, K, varied from two to four with 10 independent runs for each. Replicate runs were merged and visualized using CLUMPAK (Kopelman et al., 2015).

3. Results

Forty-one unique mitochondrial NADH2 haplotypes that are defined by 39 polymorphic sites were detected across the 128 sampled individuals (53 *C. galapagensis*, 14 haplotypes, 9 polymorphic sites; 75 *C. obscurus*, 29 haplotypes, 29 polymorphic sites). Haplotype diversity was 0.848 ± 0.030 for *C. galapagensis* and 0.904 ± 0.024 for *C. obscurus* (0.893 ± 0.020 combined). Nucleotide diversity was 0.004 ± 0.002 for *C. galapagensis* and 0.005 ± 0.003 for *C. obscurus* (0.005 ± 0.003 combined).

Carcharhinus galapagensis and *C. obscurus* were not distinguished by hierarchical AMOVA of the mitochondrial data ($F_{CT} = -0.14$), while there was substantial divergence between ocean basins ($F_{CT} = 0.23$; Table S4).

Haplotypes were linked together by 14 or fewer mutations. The network consisted of two clusters that were separated by six mutations (Fig. 2a). These clusters largely corresponded to haplotypes sampled within the Atlantic Ocean basin versus the Indo-Pacific (Fig. 2a), except for two individuals nominally identified as *C. obscurus* that were sampled from Atlantic waters off Senegal and France that clustered in the otherwise Indo-Pacific clade (Figs. 2a and S1). Haplotypes were generally closely related within the Atlantic cluster, separated by four or fewer mutations. The Atlantic

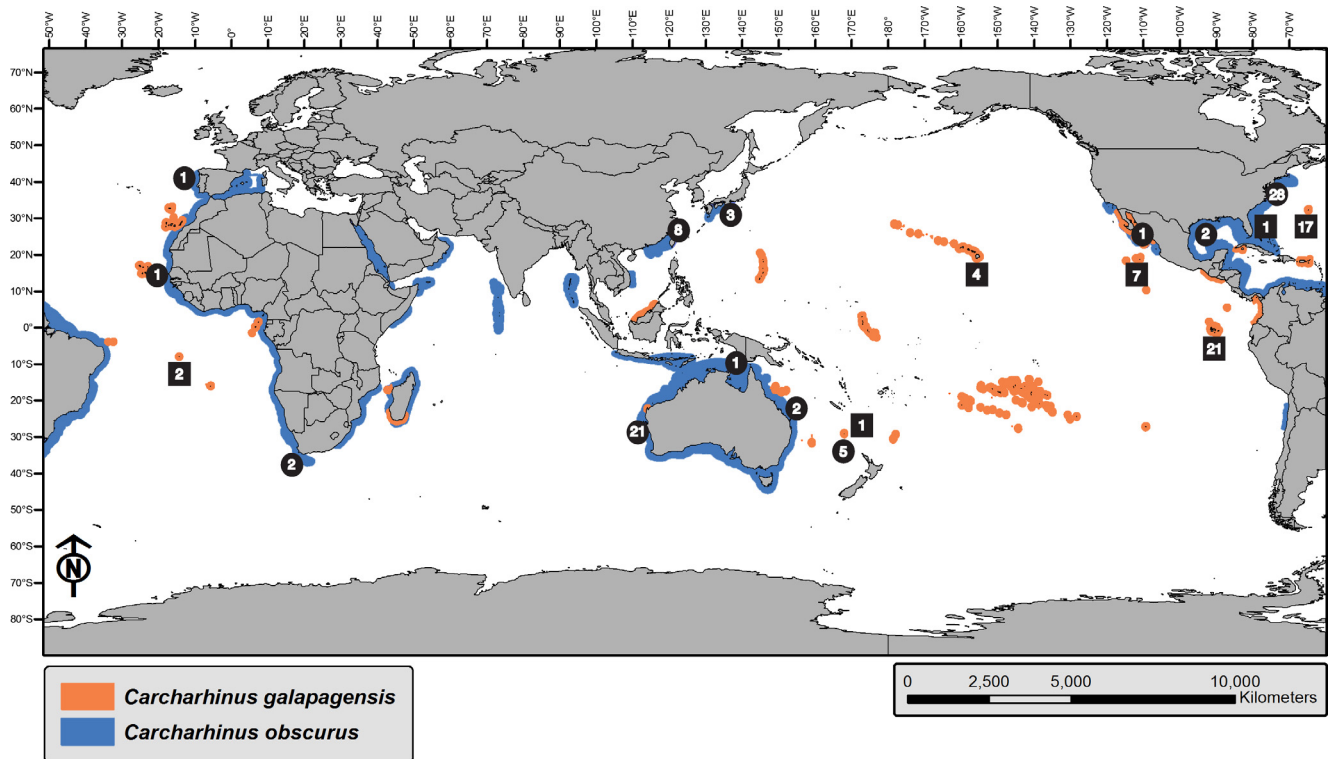


Fig. 1. Distribution ranges of *Carcharhinus galapagensis* (orange) and *C. obscurus* (blue). Sampling sites are indicated by black squares (*C. galapagensis*) and black circles (*C. obscurus*) that give the number of individuals sampled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

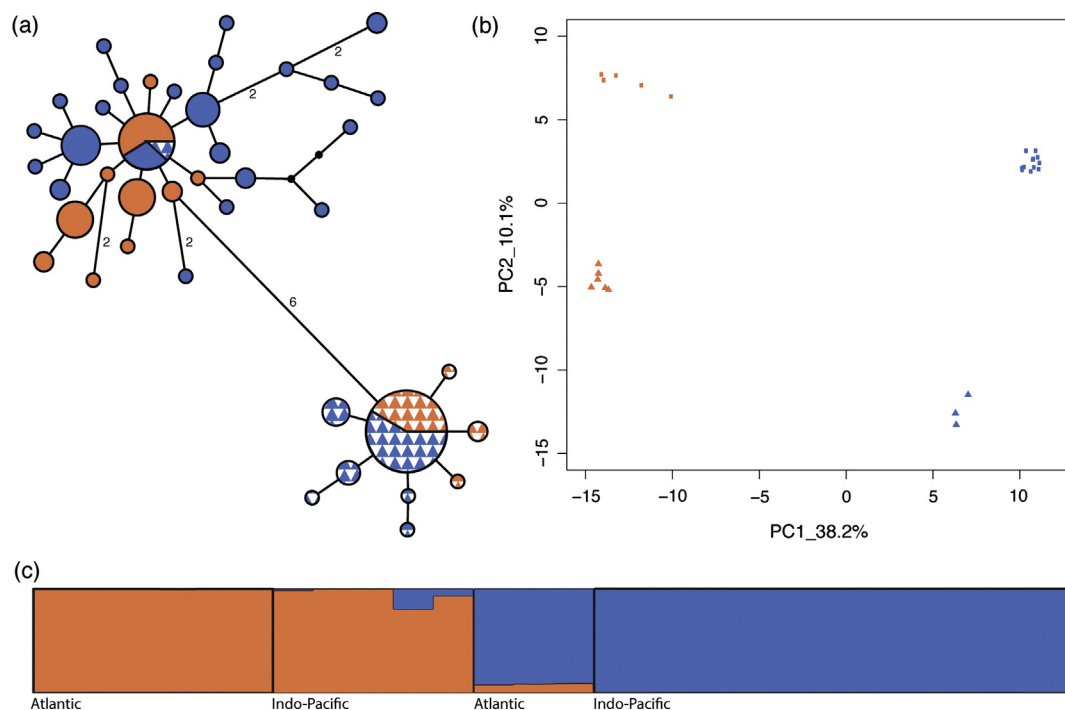


Fig. 2. (a) Median joining network of mitochondrial DNA NADH2 haplotypes (1044 bp). Superfluous links have been removed based on parsimony. The area of each haplotype circle is proportional to its frequency. Small solid black circles are intermediate states that were not observed. Numbers indicate mutational steps between haplotypes (one, if not shown). *Carcharhinus galapagensis* is in orange and *C. obscurus* in blue. The Indo-Pacific is indicated by solid colors and the Atlantic Ocean by triangles. (b) Principal components analysis based on 2152 nuclear SNPs showing PC1 and PC2. *Carcharhinus galapagensis* is in orange and *C. obscurus* in blue. The Indo-Pacific is indicated by squares and the Atlantic Ocean by triangles. (c) Plot of the estimated membership coefficients for each individual in each of two clusters (K = 2), as inferred using the program STRUCTURE (Pritchard et al., 2000) based on 2152 nuclear SNPs. Each individual is represented by a vertical column. Individuals are grouped by sampling location and colored by species. *Carcharhinus galapagensis* is in orange and *C. obscurus* in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cluster was dominated by one abundant haplotype that was sampled at all Atlantic locations, except for off of Senegal and France (Fig. S1). The remaining haplotypes in the cluster were singletons, or were associated with a small number of individuals from distinct locations (Fig. S1). The Indo-Pacific cluster was more diverse (Fig. 2a), but also contained a predominant haplotype that was sampled from most Indo-Pacific locations (Fig. S1). Four other haplotypes were encountered across multiple locations. The remaining haplotypes were singletons, or were unique to a single location (Fig. S1). Haplotypes within the Indo-Pacific clade were separated by ten or fewer mutations (Fig. 2a).

The two most abundant haplotypes in the network were shared by *C. galapagensis* and *C. obscurus*. No other haplotypes were shared among species (Fig. 2a); however, the Indo-Pacific and Atlantic clades consisted of individuals from both species. While haplotypes did not cluster according to species, ocean basins were readily distinguishable (Fig. 2a). The two clades corresponding to ocean basins were also resolved with high bootstrap support in the maximum likelihood analysis, while there was no statistical support for the separation of the two species (Fig. S2).

In contrast to the results based on the mitochondrial data, *C. galapagensis* and *C. obscurus* were readily distinguishable by hierarchical AMOVA of the 2152 nuclear SNP dataset ($F_{CT} = 0.27$). A substantial proportion of the nuclear variation was also due to differences between ocean basins within species ($F_{SC} = 0.27$; Table S4).

The first two components in the PCA explained ~50% of the variance and indicated four distinct clusters (Fig. 2b). The first principal component (PC1) separated *C. galapagensis* from *C. obscurus*, while the second principal component (PC2) showed geographical clustering by ocean basin (Fig. 2b). The four clusters persisted when considering the third (PC3) and fourth (PC4) principal components (Fig. S3). Although only accounting for approximately 10% of the total variation, the pattern in PC2 suggests greater similarity between species within ocean basins, than among ocean basins within species (Figs. 2b and S3).

The cluster analysis also supported separation of *C. galapagensis* and *C. obscurus*. The mean estimated log probability of the data was highest for $K = 2$ ($\ln \Pr(X|K) = -20877.90$), which corresponded to each of the species (Figs. 2c and S5). A small amount of admixture was observed in *C. galapagensis* from the Indo-Pacific and *C. obscurus* from the Atlantic (Figs. 2c and S5).

The *C. galapagensis* and *C. obscurus* individuals that were sampled in sympatry at Norfolk Island, and the individual sampled off Senegal, were all assigned to their nominal species clusters in both the PCA and cluster analyses (Figs. S4 and S5).

4. Discussion

Genomic-scale surveys of DNA variation are becoming more common. It is anticipated that genomic-scale sampling should reduce uncertainty arising from coalescent variation among markers (Ballard and Whitlock, 2004; Maddison, 1997). The data deluge that has accompanied the genomics era has also generated a wider appreciation of the potential for conflicting patterns of differentiation between the mitochondrial and nuclear genomes that are not the consequence of incomplete lineage sorting (ILS), but rather caused by factors such as selective differences, demographic asymmetries or hybridization (Hudson and Turelli, 2003). A recent review revealed 126 such cases in animal systems spanning a taxonomic diversity that included invertebrates, amphibians, reptiles, birds, mammals and bony fishes (Toews and Brelsford, 2012).

Here, we describe a case of widespread discordance in signal obtained from the mitochondrial versus nuclear genomes in elasmobranchs. Mitochondrial NADH2 haplotype sharing is ubiquitous

between *C. galapagensis* and *C. obscurus* (Figs. 2a and S2). The two most frequent haplotypes were shared among 27 *C. galapagensis* and 26 *C. obscurus* sampled from across their distribution ranges. In fact, interspecific mitochondrial divergence is lower than the divergence across ocean basins (Fig. 2a, Table S4). One interpretation of this pattern could be that *C. galapagensis* is the oceanic form of *C. obscurus*. However, the results of our analyses of more than 2000 nuclear SNP loci are consistent with the existing taxonomy. *Carcharhinus galapagensis* is readily distinguishable from *C. obscurus* according to the results of the AMOVA (Table S4), PCA and cluster analysis (Figs. 2b and c) of the nuclear data. Intraspecific nuclear population structure was also detected by the AMOVA and PCA, showing a clear separation between the Atlantic Ocean and Indo-Pacific samples in both species (Fig. 2b, Table S4). The individuals sampled at Norfolk Island, which represent the only location where the two species are known to occur in sympatry, were all assigned to their nominal species and ocean basin clusters, as was the individual sampled off Senegal which possesses a mitochondrial haplotype that clustered with the Pacific Ocean clade (Figs. S4 and S5).

There are several possible causes of the admixed mitochondrial signal that we observe, including ILS, and adaptive or biased mitochondrial introgression. In this case, it does not appear that the observed discordance is the consequence of ILS. Mitochondrial lineages are expected to sort more rapidly than nuclear lineages, given the four-fold smaller effective population size of the mitochondrial genome. Contrary to this expectation, nuclear lineages in our dataset appear to be sorted among species while mitochondrial lineages are not. Moreover, ILS typically results in unpredictable geographic patterns of allele sharing (Toews and Brelsford, 2012). Rather, we observe spatially widespread discordance together with a strong geographic pattern of sorting by ocean basin.

The mitochondria from one species may be adaptively introgressed into another if selection favors a particular mitochondrial variant (Funk and Omland, 2003). Given that we observe two distinct clusters of mitochondrial variants, it seems unlikely that adaptive introgression would be the sole cause of the mitochondrial admixture, as this would imply selection pressure for alternative mitochondrial variants in each ocean basin.

Particularities of the spatial dynamics and demography of species can also result in biased mitochondrial introgression, even without the influence of selection. We prefer a demographic scenario where biased introgression of mitochondrial DNA has occurred in each ocean basin during periods of secondary contact following a range expansion (Currat et al., 2008) to explain the pattern we report here. During a range expansion, alleles from the resident species can become rapidly introgressed into the expanding species during the period of demographic growth that follows colonization. When intraspecific gene flow is ongoing between the newly colonized population and others in the expanding species, the introgression will usually be biased toward markers with lower rates of migration, in this case the mitochondrial genome. These markers are more readily introgressed under such circumstances because they are less likely to be swamped by intraspecific gene flow and therefore more likely to increase in frequency due to genetic drift (Currat et al., 2008; Petit and Excoffier, 2009).

Although contemporary *C. galapagensis* and *C. obscurus* exhibit niche separation, we hypothesize that these species came into secondary contact following speciation at some point in the past, allowing their mitochondrial genomes to become biasedly introgressed within each ocean basin. Moreover, male-biased dispersal accentuates biased mitochondrial introgression (Petit and Excoffier, 2009). While male-biased dispersal has not been demonstrated in either species specifically, it is common in sharks (Dudgeon et al., 2012). Further supporting the idea that the admixed mitochondrial pattern is the consequence of historical

hybridization events is the fact that only the most frequent NADH2 haplotypes, which are typically inferred to be ancestral, are shared between species. This suggests that hybridization occurred, allowing the mitochondria of *C. galapagensis* and *C. obscurus* to become admixed, after which the species became isolated once more and resumed independent evolutionary trajectories.

We did not observe any evidence of ongoing hybridization between species in the nuclear SNP data, though we do not exclude this as a possibility upon obtaining nuclear data from a larger number of samples of both species, particularly from regions where they are sympatric. *Carcharhinus galapagensis* from the Indo-Pacific and *C. obscurus* from the Atlantic, however, did show a small amount of admixture in the cluster analysis (Figs. 2c and S5). Consistent with this, the pattern observed for PC2 in the PCA suggests that for some loci there is less interspecific variation within an ocean basin, than intraspecific variation across ocean basins (Figs. 2b and S3). We used AMOVA to test every locus for a signature of introgression that may be driving these patterns. Briefly, two separate hierarchical AMOVA were performed, for each locus, grouping the four populations (*C. galapagensis* from the Atlantic, *C. galapagensis* from Indo-Pacific, *C. obscurus* from the Atlantic and *C. obscurus* from the Indo-Pacific) by species or by ocean basin. The basic premise of this test was that introgressed loci should show lower divergence among species groups ($F_{CT\text{species}}$) than among ocean basin groups ($F_{CT\text{oceans}}$; see [Supplementary material](#) for details). Using this approach, 53 loci were identified as potentially introgressed, falling outside the range of the overall distribution of interspecific and among ocean basin divergences (Fig. S6). Presumably these loci are driving the subtle signature of admixture that we observed in the PCA and cluster analysis, and possibly represent the nuclear remnants of the same historical introgression event that resulted in the observed widespread mitochondrial admixture.

Distinguishing between alternative drivers of introgression is challenging. It is possible that a combination of selection and demography have played a role in generating the patterns that we observe. A comprehensive phylogeographic study of both species that includes extensive phenotypic and environmental data, aimed at elucidating the population and demographic histories of *C. galapagensis* and *C. obscurus*, will be required to distinguish the evolutionary processes that may have been responsible, in addition to the location and direction of historical hybridization events.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2017.03.011>.

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