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A Genetic Evaluation of the Overexploited Galápagos Sailfin Grouper, 'Bacalao' *(Mycteroperca olfax)* Fishery

Tesis de Maestría

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DEDICATORIA

I dedicate my thesis project to my amazing and supportive family- Jill, Troy, and Madeline Ferone, as well as J&J Garrison. I am grateful for everything you all have done and continue to do for me. Love you endlessly

-G

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RESUMEN

El Bacalao (*Mycteroperca olfax*), endémico de las Islas del Pacífico Tropical Oriental de Galápagos (Ecuador), Cocos (Costa Rica) y Malpelo (Colombia), enfrenta una disminución poblacional con una reducción del 30% durante las últimas cuatro décadas en la reserva marina de Galápagos y ahora está catalogado como "Endangered" por la UICN. Para informar estrategias de conservación, analizamos la diversidad genética y estructura poblacional utilizando SNPs y marcadores mitocondriales (Cytb, COI) de 84 individuos a través de seis regiones. Las redes de haplotipos de Cytb proporcionaron métricas informativas de diversidad. Los índices de fijación revelaron divergencia significativa entre el oeste de Galápagos central (F_{ST} = 0,033), sugiriendo que la Isla Isabela es una población filogenéticamente distinta. A pesar de 1000 km de separación, Malpelo y Galápagos exhibieron alta conectividad (F_{ST} = 0,002), pero Malpelo mostró señales de endogamia (FIS = 0,084) y diversidad genética reducida. El bajo tamaño efectivo de población general (N_e = 399) indica vulnerabilidad a la sobrepesca, presiones selectivas y deriva genética. Las corrientes oceánicas circulantes probablemente moldearon linajes evolutivos distintos de *M. olfax* a través de Galápagos. Dada la vulnerabilidad de la especie a la deriva genética y evidencia de patrones de dispersión masculina, el manejo de conservación debería desarrollar restricciones de pesca durante las temporadas reproductivas (octubre-diciembre) para mejorar la reproducción y la diversidad genética.

Palabras clave: SNPs | Biodiversidad | Geneticas | *Mycteroperca olfax* | Peces | Galapagos

ABSTRACT

The Galápagos Sailfin Grouper (*Mycteroperca olfax*), endemic to the Eastern Tropical Pacific Islands of Galápagos (Ecuador), Cocos (Costa Rica), and Malpelo (Colombia), faces population decline with a 30% reduction over the past four decades in the Galápagos Marine Reserve, and is now listed as Endangered by the IUCN. To inform conservation strategies, we analyzed genetic diversity and population structure using SNPs and mitochondrial markers (Cytb, COI) from 84 individuals across six regions. Haplotype networks of Cytb provided informative diversity metrics. Fixation indexes revealed significant divergence between western and southeastern bioregions of the archipelago (F_{ST} = 0.033), suggesting Isabela Island is a phylogenetically distinct population. Despite approximately 1000 km separation, Malpelo and Galápagos exhibited high connectivity (F_{ST} = 0.002), but Malpelo showed signs of inbreeding (FIS = 0.084) and reduced genetic diversity. Overall low effective population size (N_e = 399) indicates vulnerability to overfishing, selective pressures, and genetic drift. Circulating ocean currents likely shaped distinct *M. olfax* evolutionary lineages across the Galápagos. Given the species' vulnerability to genetic drift and evidence of different dispersal patterns between males and females, conservation management should develop fishing restrictions during peak spawning seasons (October-December) to enhance reproduction and promote genetic diversity.

Keywords: *Mycteroperca olfax* | Overfishing | Fisheries | Galapagos | Genetics | SNPs

TABLA DE CONTENIDO

A Genetic Evaluation of the Over-Exploited Galapagos Sailfin-Grouper 'Bacalao' *(Mycteroperca olfax)* Fishery.

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Abstract

The Galápagos Sailfin Grouper (*Mycteroperca olfax*), endemic to the Eastern Tropical Pacific Islands of Galápagos (Ecuador), Cocos (Costa Rica), and Malpelo (Colombia), faces population decline with a 30% reduction over the past four decades in the Galápagos Marine Reserve, and is now listed as Endangered by the IUCN. To inform conservation strategies, we analyzed genetic diversity and population structure using SNPs and mitochondrial markers (Cytb, COI) from 84 individuals across six regions. Haplotype networks of Cytb provided informative diversity metrics. Fixation indexes revealed significant divergence between western and southeastern bioregions of the archipelago (F_{ST} = 0.033), suggesting Isabela Island is a phylogenetically distinct population. Despite approximately 1000 km separation, Malpelo and Galápagos exhibited high connectivity (F_{ST} = 0.002), but Malpelo showed signs of inbreeding (FIS = 0.084) and reduced genetic diversity. Overall low effective population size (N_e = 399) indicates vulnerability to overfishing, selective pressures, and genetic drift. Circulating ocean currents likely shaped distinct *M. olfax* evolutionary lineages across the Galápagos. Given the species' vulnerability to genetic drift and evidence of different dispersal patterns between males and females, conservation management should develop fishing restrictions during peak spawning seasons (October-December) to enhance reproduction and promote genetic diversity.

Keywords: *Mycteroperca olfax* | Overfishing | Fisheries | Galapagos | Genetics | SNPs

Introduction

The Galápagos archipelago (Ecuador) harbors unique physical habitat structures and coral reef ecosystems for several grouper fishes belonging to the family *Serranidae*. The Galápagos Sailfin Grouper, locally named ‘bacalao,’ (not to be confused with the Spanish name for Atlantic cod *Gadus morhua*) is a keystone fish species endemic to three volcanic island groups of the ETP: the Galápagos Islands of Ecuador, Malpelo Island of Colombia, and Cocos Island of Costa Rica (Usseglio et al. 2016;

Pontón-Cevallos et al. 2020). Due to its restricted habitat range and severe overexploitation in Galápagos, the International Union for Conservation of Nature (IUCN) classified the species as *threatened* in 2022, but has since then shifted to *endangered* (Pontón-Cevallos et al. 2020; Usseglio et al. 2016; Eddy et al. 2019). Historical catch data points to a 30% population decline in the Galápagos Marine Reserve (GMR) over the past 40 years (Pontón-Cevallos et al. 2020) as bacalao are regularly fished in the GMR year-around, to be sold locally and in international markets (Usseglio et al. 2016). The Ecuadorian government classifies bacalao as "pesca blanca" (white fishes; Benavides et al. 2019), a category without regulations for the artisanal fleets to follow despite these drops in catch-yield reported by fishermen and scientists (M. Yepéz per comms., 2023). Leading up to Easter each year (March to April), there is an increase in fishing pressure (Usseglio et al. 2016); given that salted bacalao is part of a traditional Ecuadorian Easter dish called 'fanesca.' In addition to their fishery being one of the most economically important commercial markets in the Galápagos Islands (Usseglio et al. 2016), bacalao are high-trophic level fishes and ecosystem engineers that regulate the trophic pyramid (Verity et al. 2002; Okey 2004).

Bacalao (from here on referred to as *M. olfax*) grow to a very large body size (around 1-meter length), live a long time (20-30 years), mature slowly (size at first reproduction: 65.3 cm), are protogynous hermaphrodites (born as female and change to male later in life), and demonstrate spawning aggregation behaviors that allow targeted capturing of reproductive individuals (Sadovy 1996, 2005; Pontón-Cevallos et al. 2020). The *Serranidae* family of fishes generally take a long time to reach population carrying capacity; depending on the site conditions it can take 2 to 4 decades after the implementation of no-take reserves (Abesamis et al. 2014). A loss in genetic diversity, inbreeding depressions, and mutation accumulations have previously been shown to increase extinction risk (Frankham 2005); and furthermore, studies suggest that small and isolated populations of fishes are at higher risk of extinction due to inbreeding, reduced immigration, and low genetic diversity (Dulvy et al. 2003, 2004). These characteristics can catalyze the extinction vortex where seasonal die-offs occur frequently and at an increasing rate (Blomqvist et al. 2010). Low genetic diversity can also increase marine fishes' vulnerability to genetic drift events due to the fragility and uniqueness of locally adapted

77 gene pools (Carvalho 1993); implying that adaptive pressures like selective fishing, climate change, and
78 habitat degradation can lead isolated or inbred populations towards extinction faster than healthy, diverse,
79 and connected ones (Frankham 2005).
80 *Mycteroperca olfax* are aggregation spawners, which means that they breed in groups either
81 through necessity, limited resources, or evolutionary benefits for reproduction (Molloy et al. 2012).
82 Spawning events generally occur from October to December in areas of residence, or at well-defined
83 pelagic aggregation sites, sometimes kilometers away from home reefs (Sadovy 1996; Molloy et al. 2012;
84 Usseglio et al. 2015). Many marine fish and invertebrates produce small young that are too weak to swim
85 and are therefore carried by ocean currents; this can result in longer dispersal distances of 100s-1000s of
86 kilometers (Metaxas et al. 2024). Since the ETP is characterized by a complex interplay of ocean currents
87 that influence the dispersal of marine organisms and particularly grouper fishes (Montecino and Lange
88 2009; Jakoboski et al. 2020), the distribution, transport, and fertilization of eggs relies heavily on the
89 location of aggregation sites and the dispersal of male broadcast spawners (Phillips et al. 2021; Chong-
90 Montenegro and Kindsvater 2022). Meanwhile, the ETP's major currents (i.e., the North and South
91 Equatorial currents, Peru current, Panama current, and Humboldt current) create eddies, upwelling zones,
92 and areas of stratification around islands (Montecino and Lange 2009). Convergences of multi-directional
93 currents can either facilitate or impede fish larval dispersal among islands like Galápagos, Malpelo,
94 Revillagigedo, and Cocos (Montecino and Lange 2009). The delivery of eggs and larvae from *Serranidae*
95 species around the ETP islands are likely driven by these circulations, and extreme events like El Niño
96 Southern Oscillation (ENSO) which disrupt normal patterns (Arntz et al. 2006; Edgar et al. 2010; Wood
97 et al. 2016). Larval dispersal has been shown to drive genetic and morphological isolation in reef-fishes
98 (Cowen et al. 2006), and furthermore, widespread larval dispersal throughout the ocean can obscure
99 genetic differentiation between subgroups (Kough et al. 2013). The western GMR (Site 7, Fig. 1), is a
100 unique bioregion that harbors high rates of endemism and primary productivity (Edgar et al. 2004; Kislik
101 et al. 2017). However, this area's protection from strong currents like the Humboldt or Panama, and thus

its lack of exposure to larvae and egg deposits, raises questions about the genetic influx of reef-fishes like *M. olfax* to the region.

To implement conservation protocols for the endangered grouper, *M. olfax,* the information provided by molecular tools (i.e., Hardy-Weinberg Equilibrium analysis, genetic stock identification or connectivity, and effective population size (N_e) estimation) is necessary considering each regional stock requires unique management strategies depending on the species' reproductive connectivity, population genetics history, and dispersal (Pazmiño et al. 2017). Calculating observed heterozygosity and its comparison to HWE may reveal selective pressures, inbreeding, or stratification affecting populations (Wigginton et al. 2005). Genetic stock assignment (K-cluster analysis) and F_{ST} (fixation) indexing, which quantify connectivity and reproductive isolation (Begg et al. 1999), and N_e estimations that combine genetic diversity metrics with the life history of *M. olfax*, may be used to predict the current and future viability of the species (Hare et al. 2011). These metrics in conjunction with mitochondrial analyses may illustrate the life history and current genetic health of *M. olfax*, and provide insights into behaviors, reproduction, and dispersal (Pazmiño et al. 2017). Specifically, discrepancies between mtDNA metrics and nuclear DNA (nDNA) results, can reveal sex-based differences in reproduction and dispersal patterns in hermaphroditic fish (Phillips et al. 2021; Chong-Montenegro and Kindsvater 2022).

This study elucidated the genetic composition and population genetics history of *M. olfax*, while establishing a baseline of genetic sequences for future monitoring and research. Here, we aimed to reveal genetic health parameters useful for guiding the conservation of reef-fish in the GMR by deploying the following pieces of information: (1) mitochondrial Cytb and nuclear SNPs screening, to compare the outputs and population structures resolved by both methods; (2) revealing the determiners of population health like regional observed heterozygosity (H_o) and comparisons to expected heterozygosity under HWE; (3) mapping connectivity and gene flow of *M. oflax* throughout the ETP via fixation index, and exploring reproductive isolation and its importance to the conservation of fishes; and (4) estimating effective population size to predict the resilience of *M. olfax* in the GMR for use by conservation managers and government agencies.

Materials and Methods

Permission to carry out sampling methods and laboratory procedures was acquired through permit: #MAATE-DBI-CM-2021-0174: *Código de barras génetico de Galápagos exploración genética para el studio de la evolución* granted by the Ecuadorian Ministry of Environment. A total of 119 individuals were caught and sampled from 10 different locations across 6 bioregions: Malpelo (18), Darwin & Wolf (15), San Cristóbal (53), Santa Cruz (12), Española (10) and Isabela (11). Capture and sampling locations of *M. olfax* from the field work (June 2023–August 2024) are shown in Fig. 1.

Individuals of *M. olfax* were caught using hand lines called 'empates' (M. Yepez & Y. Revelo pers. comm., 2024; weighted rig with a drop line and hook) around the island of Española (-1.3667, -89.6667), taken from fishermen at the San Cristóbal Puerto Baquerizo Moreno (-0.89870, -89.61170), in a boat with fishermen at Punta Pitt (-0.54083, -89.36302), Bahía Sardina (-0.69714, -89.362333), Isabela (-0.965847, -90.9791), and Santa Cruz (-0.744866, -90.3079), during an expedition at Darwin Island (1.67089, -92.00878), and sent to Quito (Ecuador) from Malpelo Island (4.0000, -81.5850). Samples were stored at −20°C in the Galapagos Science Center prior to extraction and Polymerase Chain Reaction (PCR). Muscle tissue samples were processed over the summer (June–August) of 2024 at the Microbiology and Molecular laboratory of the Galápagos Science Center in San Cristóbal Galápagos. DNA was extracted using 'Qiagen DNeasy Blood and Tissue Kits' (Qiagen, Valencia, CA). All samples following DNA extraction were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) to verify the concentration and contamination levels of the extractions. Mitochondrial DNA markers have previously been used to study the population genetics structure and differentiation of marine fishes in the Galápagos (Sulaiman et al. 2021). COI and Cytochrome b (Cytb) primers, were chosen for their utility in identifying fish species, and also since previous work described COI and Cytb as successful genes for sequencing fishes of various families (Sulaiman et al. 2021). The COI gene has been widely used as a DNA barcode for fish identification, while the Cytb gene has been shown to provide higher resolution for distinguishing closely related species and for haplotype extraction

and networking (Ma et al. 2020). Amplification by PCR was performed on all 119 samples using these two genes. The primers used for the COI and Cytb amplifications, their base pair lengths, and additional amplification protocol information are provided in Table S1. COI and Cytb sequences were obtained via an ABI capillary electrophoresis system sequencer using PCR primers, through Macrogen Labs (Seoul, South Korea). Sequence chromatograms were aligned using Geneious Prime (Geneious Prime 2025.0.1) De-novo assembly function. A phylogenetic tree was estimated using Randomized Accelerated Maximum Likelihood (RAxML) phylogenetic tree assemblage, also in Geneious Prime, and bootstrap analysis of 1000 replicates was conducted to assess branch support values. The best-scoring ML tree was visualized with bootstrap support values displayed at key nodes. Only sequences falling above 97% HQ read quality were used for subsequent mitochondrial analysis (n = 119). The BLAST function in Geneious Prime for each sample verified M. olfax species with high percentage matches. Nucleotide diversities were calculated using the Cytb gene alignments with the 'DnaSP' package (Rozas and Rozas 1997) in R (R Core Team 2025), and haplotype networks were calculated and visualized a minimum spanning network using POPART software (Leigh and Bryant 2015) along with the 'ape' R-package in R-Studio (Paradis and Schliep 2019). Haplotype statistics were also re-calculated using the following equations: Haplotype Diversity (Hd): $Hd = (1 - \sum x_i^2) \times (n/(n-1))$; where: n = total number of sequences in the sample; x_i = frequency of the i'th haplotype; $\sum x_i^2$ = sum of the squared haplotype frequencies.

DArT sequencing (Diversity Arrays Technology sequencing, Canberra, Australia) is a high-throughput genotyping method used to discover and score single nucleotide polymorphisms (SNPs) and presence/absence markers across genomes. The genomic DNA from each sample was digested using restriction enzymes by targeting specific regions for sequencing, to identify low-copy genomic regions more likely to contain useful variation. We implemented filtering steps to obtain high-quality SNPs for nuclear DNA analyses. We removed duplicate SNPs located on the same DNA sequence fragment (i.e., tightly linked loci) and retained only the SNPs with the highest average reproducibility, polymorphism information content (PIC), and average read depth per fragment. We eliminated monomorphic loci, which were either homozygous or heterozygous across all sampled individuals and thus uninformative for

population structure inference. We implemented an iterative filtering approach for call rate to remove low-quality loci using a threshold of 0.85. We only retained individuals with call rates exceeding 0.90. Individuals exhibiting excessively high heterozygosity levels were excluded from the dataset as potentially contaminated samples. Following individual filtering, we performed a second round of monomorphic locus removal. To ensure marker reliability, we removed SNPs with reproducibility values below 0.95, based on technical replicates included in the DArTseq library preparation (Gruber et al. 2018). Low-frequency alleles were filtered by implementing a minor allele frequency (MAF) threshold of 0.02. We eliminated SNPs with excessive heterozygosity, which could indicate the presence of paralogous sequences or sequencing errors. We employed two complementary outlier detection methods in R-studio to detect extreme loci. First we applied OutFLANK (Whitlock and Lotterhos 2015), which uses F_{ST} distributions to detect loci with unusually high differentiation compared to the genomic background. Next, we utilized PCadapt (Luu et al. 2017) which identifies outliers based on their association with population structure using principal component analysis. We combined results from both methods to our dataset of 4,832 SNPs and identified 46 putative outlier loci: OutFLANK (11), PCadapt (35). These outlier loci were subsequently removed from the primary dataset to create a neutral SNP dataset containing 84 individuals with 4,786 loci, which was used for demographic analyses. We also retained the 46 identified outlier loci in a separate table for selection analyses.

Estimations of N_e for each cluster were done using the molecular co-ancestry method of *Nomura 2008,* as implemented in NeEstimator V2.1 (Do et al. 2014). Fixation indexes, discriminant analysis (DAPC), and group stock assignments (to determine N_e groups), were generated following applications from *Nikolic et al. 2023.* For contemporary estimations of N_e as outlined by *Do et al. 2014,* a minimum sample size of 27 is recommended for Linkage Disequilibrium methods, and LD methods are also documented as being the best performing calculators for N_e regardless of sample size (Gilbert and Whitlock 2015). Observed and expected heterozygosity (H_o and H_e) as well as F_{ST} calculations were all performed using the R Studio package Adegenet (Jombart and Ahmed 2011).

Results

mtDNA Results

Mitochondrial Cytb sequences (760 base-pair, bp) lengths were analyzed for 119 samples (Fig. 2 and Fig. S1). Cytb produced more differentiation than COI (650 bp), suggesting it is a more favorable gene for studying population level differences in *M. olfax*. Cytb produced defined grouping clusters (Fig. 2), including one with BC501, BC512, BC516 (Darwin & Wolf), and BC528 (Mapelo), suggesting divergence of samples from the north and eastern bioregions. Also, the cluster of BC533, BC536, BC537 (Malpelo), and BC495 (Santa Cruz) indicating a paired divergence in the central GMR region as well, combined with Malpelo.

Haplotype network analysis for Cytb with 119 *M. olfax* samples (Fig. 3), revealed 11 distinct haplotypes: four shared and seven unique haplotypes. Collectively, H6 and H10 included most of the samples and represented all 6 sampling locations (n = 64 and n = 44). H9 was shared between Malpelo and Darwin & Wolf, and H11 was shared between Española and Santa Cruz. Our network structure shows three unique haplotypes for Darwin & Wolf (H1, H2, and H3), three for Malpelo (H4, H5, and H7), and one for San Cristóbal (H8). Each represented by a single individual variant. Evidence of mixing occurring between northern Galápagos and Malpelo is shown with haplotype H9, where a divergent Darwin & Wolf sample matched one from Malpelo. Overall haplotype and nucleotide diversity was 0.578 ± [0.521–0.634], and 0.0015 ± [0.0009–0.0022] respectively. In comparison, Española had the lowest haplotype diversity of h = 0.378 ± [0.289–0.467]. San Cristóbal, despite being the highest sample size (n = 53), showed a moderate h of 0.528 ± [0.509–0.547]. Isabela also showed low h and π diversity and was only represented in two haplotypes (H6 and H10).

SNPs Results

Initially 34,491 SNPs were detected across 107 *Mycteroperca olfax* individuals. During the filtering process we removed 3,450 duplicated SNPs, 7,328 monomorphic loci, 5,213 SNPs through call

rate filtering (threshold set at 0.85), and 1,439 additional loci when excluding 20 individuals with call rates below 0.90. We also excluded 5 individuals showing excessive heterozygosity, removed 3,086 newly monomorphic SNPs, 2,606 SNPs with reproducibility below 0.95, 6,509 SNPs with MAF below 0.02, and 43 SNPs with excessive heterozygosity. The resulting filtered dataset consisted of 4,832 high-quality SNPs across 84 *M. olfax* individuals, which was used for all subsequent analyses. All non-neutral loci were removed from SNPs analysis in order to optimize the accuracy and validity of H_e & H_o, F_{ST}, and N_e calculations.

Allelic richness was highest in Darwin & Wolf (1.639) followed by San Cristóbal (1.635), and lowest in Isabela (1.483). The average allelic richness across all populations was 1.568 (95% CI: 1.502–1.623). Observed heterozygosity (Ho) ranged from the lowest value of 0.151 in Malpelo to 0.203 in Darwin & Wolf, with a mean of 0.178 across all populations (95% CI: 0.173–0.180). Malpelo also generated the highest inbreeding coefficient (FIS) of 0.084, while all other populations produced negative or near-zero values for FIS. Hardy-Weinberg Equilibrium tests yielded consistent values of 1.000 across suggesting no significant deviation from H_e for all regions except Malpelo (H_o = 0.151; adjusted H_e = 0.174) which was statistically lower than expected (Table S2). Notable differentiation occurred between the west and east GMR (Fig. 4). Isabela and Santa Cruz generated the highest pairwise F_{ST} of 0.033 ($p = 0.03$), followed by Isabela and Española with 0.031 ($p = 0.01$). Darwin & Wolf deviated from Isabela ($F_{ST} = 0.026$; $p = 0.01$), and Española ($F_{ST} = 0.010$, $p < 0.05$) while Santa Cruz and San Cristóbal produced the lowest F_{ST} (ranging from 0.002-0.018, $p > 0.05$ each) suggesting high connectivity among sites in the central GMR region. Malpelo produced low F_{ST} with San Cristóbal (0.006), Santa Cruz (0.002), and Española (0.005); ($p > 0.05$ each) suggesting that more mixing occurred between Malpelo and the central GMR islands, than with Isabela (0.033 > 0.002).

The population structure analysis revealed three distinct genetic clusters (K = 3) based on 5 principal components (Fig. 5). Differentiation was confirmed again in Isabela (6 out of 7 assigned to the second genetic cluster) from the other islands. Española, Malpelo, and Santa Cruz displayed brown (cluster 1) assignments, with some admixture between them. A third genetic cluster emerged (orange/red

colored bars) in four samples at Darwin & Wolf. Calculations of N_e for K group 1 (n = 74; excluding K = 2 and K = 3) were performed using different allele frequency thresholds (0.050, 0.020, 0.010, and 0+). We revealed consistent LD metrics across thresholds, with overall r^2 values of approximately 0.0144 ± 0.0145 (± representing 95% confidence interval) and expected $r^2 = 0.0136 \pm 0.0137$. The final N_e ranged from 399.0 to 417.2. Parametric 95% confidence intervals for $N_e = 394.1 \pm 409.3$ to 404.0 ± 425.3; jackknife resampling = 244.0 ± 252.5 to 893.5 ± 1247.5.

Discussion

Genetic Connectivity

Connectivity analyses of *M. olfax* showed significant deviations in genetic structure and F_{ST} values, suggesting that western Isabela is genetically isolated ($F_{ST} = 0.018–0.033$; $p < 0.05$ each). Compared to previous studies of F_{ST} in marine fishes with high gene flow, these values demonstrate consistent fine-scale population structure despite being below 0.05 (Waples 1998; Nielsen et al. 2009). Additional F_{ST} analyses with outlier loci produced the same patterns of differentiation, however these results are to be interpreted with caution due to the lack of a reference genome. A 2017 study conducted on the Galápagos Shark (*Carcharhinus galapagensis)* discovered similar association and low connectivity in individuals from western Isabela (Pazmiño et al. 2017). This pattern parallels our findings for *M. olfax,* and further, *C. galapagensis* is a highly vagile species, which infers that Isabela's isolation may extend across sedentary and highly mobile fishes. Fishing pressure is high between Fernandina and Isabela (known as the "las Marielas" channel; Chinacalle-Martínez et al. 2024; Y. Revelo pers. comm., 2024). We expected this region of the GMR to be densely populated with *M. olfax* on the basis of Isabela's elevated sea surface temperatures, unique upwelling conditions, and concentration of endemic species (Edgar et al. 2004; Pazmiño et al. 2017; Kislik et al. 2017). These environmental characteristics have been shown to promote reef-fish productivity and biodiversity (Edgar et al. 2004). Furthermore, the aggregated spawning characteristics of *M. olfax* as explained in Sadovy (1996) and Molloy et al. (2012), likely leads

to higher connectivity amongst islands that are circulated by the Panama Current from the north coastline, and the Humboldt Current, south of the continent (Chavez and Brusca 1991; Montecino and Lange 2009). Western Isabela is positioned out of reach from these two major current systems; the only entry point of *M. olfax* larvae would be the South Equatorial Counter (Cromwell) current which runs from west to east (Chavez and Brusca 1991; Montecino and Lange 2009). Low haplotype and nucleotide diversity were also calculated in Isabela, while no significant deviations from HWE nor positive inbreeding was found through nDNA analyses. Different diversity metrics between mtDNA and nDNA, can result from different evolutionary rates, inheritance patterns, or sex based dispersal if fitness needs deviate between males and females in the population (Phillips et al. 2021). The sequential hermaphroditism of *M. olfax* (Chong-Montenegro and Kindsvater 2022) is likely the driver of highly informative Cytb results here, and suggests that higher male (than female) dispersal could be occurring in this species during reproductive months.

Malpelo is geographically isolated from the GMR by approximately 1000 km, however, Malpelo and eastern Galápagos have similarities in endemism and functional richness of reef-fishes (Quimbayo et al. 2017; Claudino-Sales 2019). Both cluster analysis and F_{ST} revealed high gene-flow and admixture occurring between Malpelo and East/Central GMR ($F_{ST} < 0.003$). Generally, *M. olfax* migrate to deeper waters during spawning seasons (October–December) and will subsequently return to their home reefs; as *M. olfax* are highly residential and reef-associated (Usseglio et al. 2015; Pontón-Cevallos et al. 2020). Since larval stages have been shown to last for weeks or even months in reef-fishes depending on water conditions (Wood et al. 2016; Metaxas et al. 2024), and the theory of large-scale-pelagic migrations of *M. olfax* is highly unlikely due to restricted habitat range (Usseglio et al. 2016), this high geneflow between Malpelo and Galápagos (excluding western Isabela) likely points towards ocean circulation as being the main driver of Malpelo-Galápagos mixing.

mtDNA Phylogeography

A skewed male to female ratio (1:1,000; Usseglio et al. 2015), could enhance the utility of Cytb for analyzing basic population structures and haplotype networks in *M. olfax* since mtDNA markers are passed down maternally (Guo et al. 2004). The distribution of 11 haplotypes across 6 major regions demonstrated patterns in population structure non congruent with separation by geographic distance. Malpelo contained three unique haplotypes which implied some genetic differentiation at this site much like Darwin & Wolf. However, we discovered that every Darwin & Wolf haplotype's nearest neighbor was from Malpelo, instead of southern Galápagos. In addition, the shared H9 haplotype could be evidence of mixing between Darwin & Wolf and Malpelo and posits that east to west ETP connectivity (i.e., Darwin & Wolf vs. Malpelo) may be stronger than the north to south gradient (i.e., Darwin & Wolf vs. Española). These findings are also supported by existing ocean circulation theories (Montecino and Lange 2009), and could support the historical gene flow between the two distant island groups. Our diversity metrics (π = 0.0014–0.002; and h = 0.378–0.705) closely matched a previous study of mtDNA for *M. olfax* in Galápagos (Sulaiman et al. 2021), which revealed that *M. olfax* Cytb had low haplotype and nucleotide diversities (π = 0.00120; and h = 0.538) and suggested this situation to be driven by overfishing.

Population Health and N_e

Malpelo exhibited the highest FIS (0.084), lowest H_o (0.151), and significant deviation from HWE, inferring that low genetic diversity could be increasing this region's venerability to selective pressures like isolation and a lack of immigrating individuals. Fishing is prohibited inside their marine protected boundary, however, high functional richness and fish biomass suggests that Malpelo is still one of the most ecologically vulnerable islands of the ETP (Quimbayo et al. 2017; Claudino-Sales 2019). Results of our F_{ST} matrix showed frequent mixing, and we argue that the flow of the Panama and Humboldt currents (Arntz et al. 2006; Montecino and Lange 2009), may be the vector of geneflow between the GMR Islands. Further analysis on directionality may explain the low genetic diversity and

high inbreeding coefficients in Malpelo, which are symbolic of small and isolated populations (Vrijenhoek 1994). Since Malpelo may serve as a key source of genetic diversity for the GMR, protecting this flow and exchange of genetic material is imperative for conserving *M. olfax* and potentially other *Serranidae* fishes throughout the ETP.

Linkage Disequilibrium calculations produced a N_e of 399.0 (95% CI: 394.1–404.0) using a combined sample size of 76 individuals (all individuals from clusters K = 2 and K = 3). The magnitude of N_e is lower than the 500 standard cut-off (considered the lowest N_e value or a population to be self-sustainable in future generations; Braude and Low 2010). This value also suggests *M. olfax* is vulnerable to anthropogenic and environmental stresses over the long run. While our N_e was not low enough to infer recent bottlenecks or genetic drift events (Braude and Low 2010), N_e of 399.0 suggests that *M. olfax* population size will continue to drop in the future unless interventions are put in place.

Conservation implications

The lack of fishing regulations over the past decades have led to major species population declines in Galápagos. For example, the Sea Cucumber *(Isostichopus fuscus*), and the Spiny Lobster *(Panulirus penicillatus)*, showed alarming rates of depletion in response to resource exploitation (Hearn 2008). Overfishing poses a significantly greater risk to species with low N_e as selective pressures can compound (Pinsky and Palumbi 2014). We argue that *M. olfax's* vulnerability to genetic drift should be of major concern to the Galápagos National Park Directorate since the artisanal fishery is an economic and cultural staple to the local people of Galápagos and continental Ecuador. With a 1,000:1 ratio of females to males, maintaining the diversity of males in the system should be a priority for conservationists (Usseglio et al. 2015; Pontón-Cevallos et al. 2020). In species with skewed sex-ratios, extracting males from the gene pool via selective fishing can halt reproduction rates and lower their genetic diversity (Usseglio et al. 2015; Pontón-Cevallos et al. 2020). Conservation actions should consider the heightened vulnerability of male *M. olfax* to selective pressures, as well as their increased importance for local

reproduction and gene flow. Fishing regulations mitigating the harvest of males (based on size-limits), could be a foundational step for their conservation. More research regarding their reproduction and life stages, would help determine this optimal size limit for *M. olfax* (Carver et al. 2005; Hamilton et al. 2007).

We revealed that frequent mixing may be occurring between Malpelo and Galápagos via the Humboldt and Panama currents, and the connectivity amongst the volcanic islands of the ETP may be essential for evolutionary succession of *M. olfax.* Understanding that Malpelo may represent a genetic source for the GMR to sustain diversity in populations of *M. olfax* can help us guide conservation efforts towards preserving the waterway between Galápagos and Malpelo. Furthermore, Malpelo exhibiting high FIS and low H_o compared to all other locations is typical of isolated subpopulations and a warning sign of population instability (Braude and Low 2010). We urge scientists to continue monitoring the genetic composition of *M. olfax* in Malpelo and understand this region's impact on the structure of the GMR. More information is needed regarding the directionality and phylogenetic history between these two island groups since our study was limited by a small sample size and lack of sampling in intermediate zones like Cocos Island. Recent MPAs and corridors have been implemented in the ETP (e.g., Hermandad Reserve) to protect pelagically migrating species like sharks and rays *(Elasmobranchii)* and to reduce the impact of industrial fishing between Cocos, Galápagos, and Malpelo (White et al. 2023). Meanwhile, studying the composition of larvae in the water channels may be a starting point for interpreting the phylogeny of endemic fishes like *M. olfax*, given that ocean circulations may influence their reproduction and distribution. Further, genetic sampling of other *Serranidae* fishes would allow to compare multispecies connectivity and structure around the ETP and promote the regional conservation of fisheries.

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Statements and Declarations

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Competing Interests

The authors have no relavent financial or non-financial interests to disclose.

Author Contributions

GSF: Directed the study, led field collection, sampling, laboratory, data analysis, and wrote manuscripts; MM: Assisted field collection (provided approximately 30% of *M. olfax* samples), assisted in manuscript revisions and project guidance; AH: Contributed to and revised manuscripts, conducted expeditions for data collection, co-advised GSF's thesis work alongside DP; EB: assisted in Mitochondrial sequencing analysis, helped direct the study, revised and reviewed final manuscripts. JMG: Reviewed manuscripts, helped design study methods, contributed to finding the project; DV: Aided in sample collection and laboratory work, reviewed and contributed to final manuscripts, helped design map figures; DP: Main supervisor of the project, advised GSF as a masters advisor, led sequencing processes, reviewed and revised manuscripts, provided funding through grants, and helped organize the study overall.

Data Availability

R-markdown files used for conducting analyses can be found publicly on GitHub through the following link: https://github.com/GarrisonFishes/BacalaoProject.

Tables

Table 1: Haplotype table information across 6 regions (119 individuals).

Population	Sample Size	Haplotypes	Haplotype Distribution	Haplotype diversity (h)	Nucleotide diversity (π)
Malpelo	18	6	H4, H5~H7, H9~H10	0.699 ± [0.672-0.726]	0.0019 ± [0.0012-0.0026]
Darwin & Wolf	15	6	H1~H3, H6, H9~H10	0.705 ± [0.676-0.734]	0.0020 ± [0.0013-0.0027]
San Cristobal	53	3	H6, H8, H10	0.528 ± [0.509-0.547]	0.0014 ± [0.0010-0.0018]
Española	10	3	H6, H10~H11	0.378 ± [0.289-0.467]	0.0009 ± [0.0005-0.0013]
Santa Cruz	12	3	H6, H10~H11	0.530 ± [0.464-0.596]	0.0015 ± [0.0008-0.0022]
Isabela	11	2	H6, H10	0.509 ± [0.404-0.614]	0.0014 ± [0.0007-0.0021]

Samples from Malpelo (18), Darwin & Wolf (15), San Cristobal (53), Española (10), Santa Cruz (12), and Isabela (11), were grouped into 11 different haplotypes. Distributions of haplotypes H1-H11 were identified for each population and used to calculate haplotype diversity and nucleotide diversity.

Figure Legends

Fig. 1: Map of the study site, Galapagos Islands and Eastern Pacific Islands. Site coordinates listed below. Site coordinates listed below; bathymetry: "Ocean Depth (m)" displayed based on the color spectrum in the legend. Economic Exclusive Zone (EEZ) and GMR are also visualized on the map. Sampling sites were as follows: (1) Puerto Baquerizo Moreno, San Cristóbal; (2) Punta Pitt, San Cristóbal; (3) Bahía Sardina, San Cristobal; (4) Puerto Ayora, Santa Cruz; (5) Española; (6) Puerto Villamil, Isabela (East); (7) Las Marielas, Isabela (West Canal); (8) Darwin; (9) Wolf; (10) Malpelo.

Fig. 2: Phylogenetic Tree using Cytb gene for 84 bacalao individuals (this abbreviated tree uses n=84). Number of bootstrap trees = 100, consensus support threshold: 100%. The full tree including all individuals (n=119) can be found in Supplementary Information (S1).

Fig. 3.1: *M. olfax* haplotype network (Minimum Spanning Network), based on informative variable characters of the mtDNA Cytb region. Circles represent each haplotype, and circle size is proportional to haplotype abundance (number of individuals). Branch lengths and line hashes represent the number of mutations between haplotypes. The color legend corresponds to the map with site numbers in Fig. 1; Red: San Cristóbal (1, 2, 3); Orange: Santa Cruz (4); Brown: Española (5); Purple: Isabela (6, 7); Blue: Darwin & Wolf (8, 9); Green: Malpelo (10).

Fig. 3.2: *M. olfax* haplotype networks (Minimum Spanning Networks), based on informative variable characters of the mtDNA Cytb region for each individual population. Each box with a haplotype network

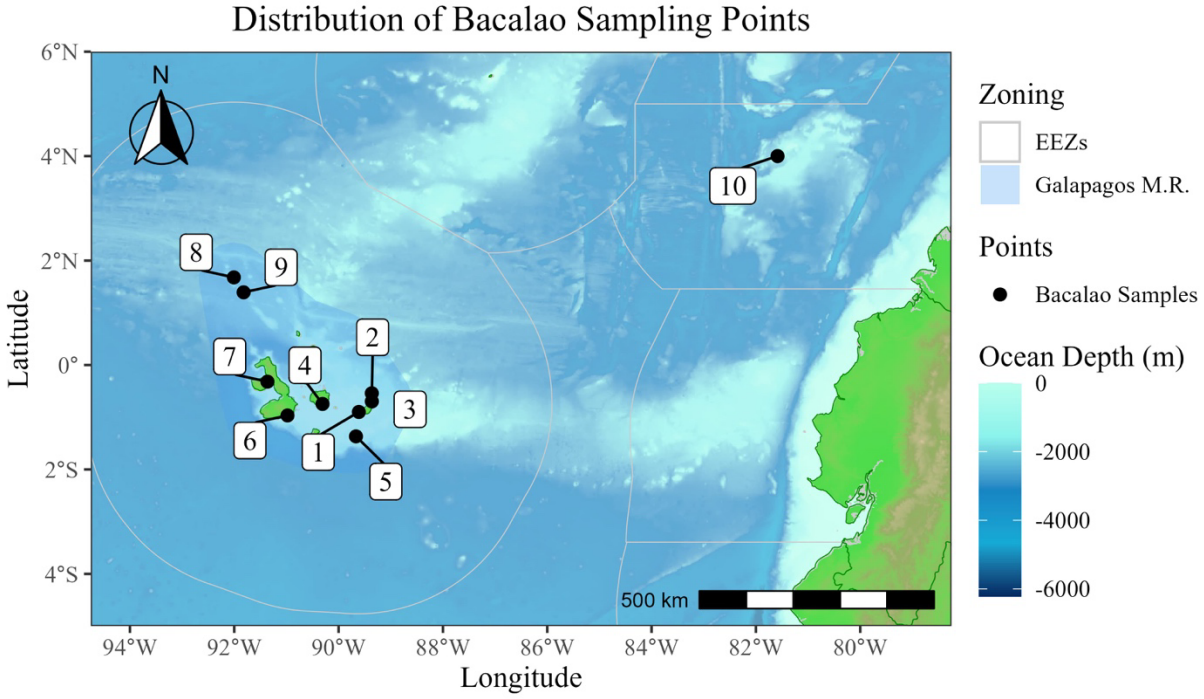
corresponds to one of the following in the map: Red: San Cristóbal (1, 2, 3); Orange: Santa Cruz (4); Brown: Española (5); Purple: Isabela (6, 7); Blue: Darwin & Wolf (8, 9); Green: Malpelo (10).

Fig. 4: Fixation heatmap: F_{ST} values (above diagonal) ranging from approximately 0.00 to 0.03, indicating the amount of differentiation between populations. Values above 0.02 (dark red) represent weaker connectivity and more genetic isolation. Values below 0.01 (white) = stronger genetic connectivity and less differentiation. P-values of Weir and Cockerham's F_{ST} calculations are listed below the diagonal in the corresponding squares.

Fig. 5: Structure plots using the R-package 'adegenet' for 84 *M. olfax* samples across 4,832 loci. Groupings were made without location priors and relied on DAPC assignments. Number of clusters was set at K=3 and PCs = 5 (achieving lowest BIC). Assignments were based on percentage matches to each cluster ranging from 0.0 – 1.0.

Figures

628 Fig. 1



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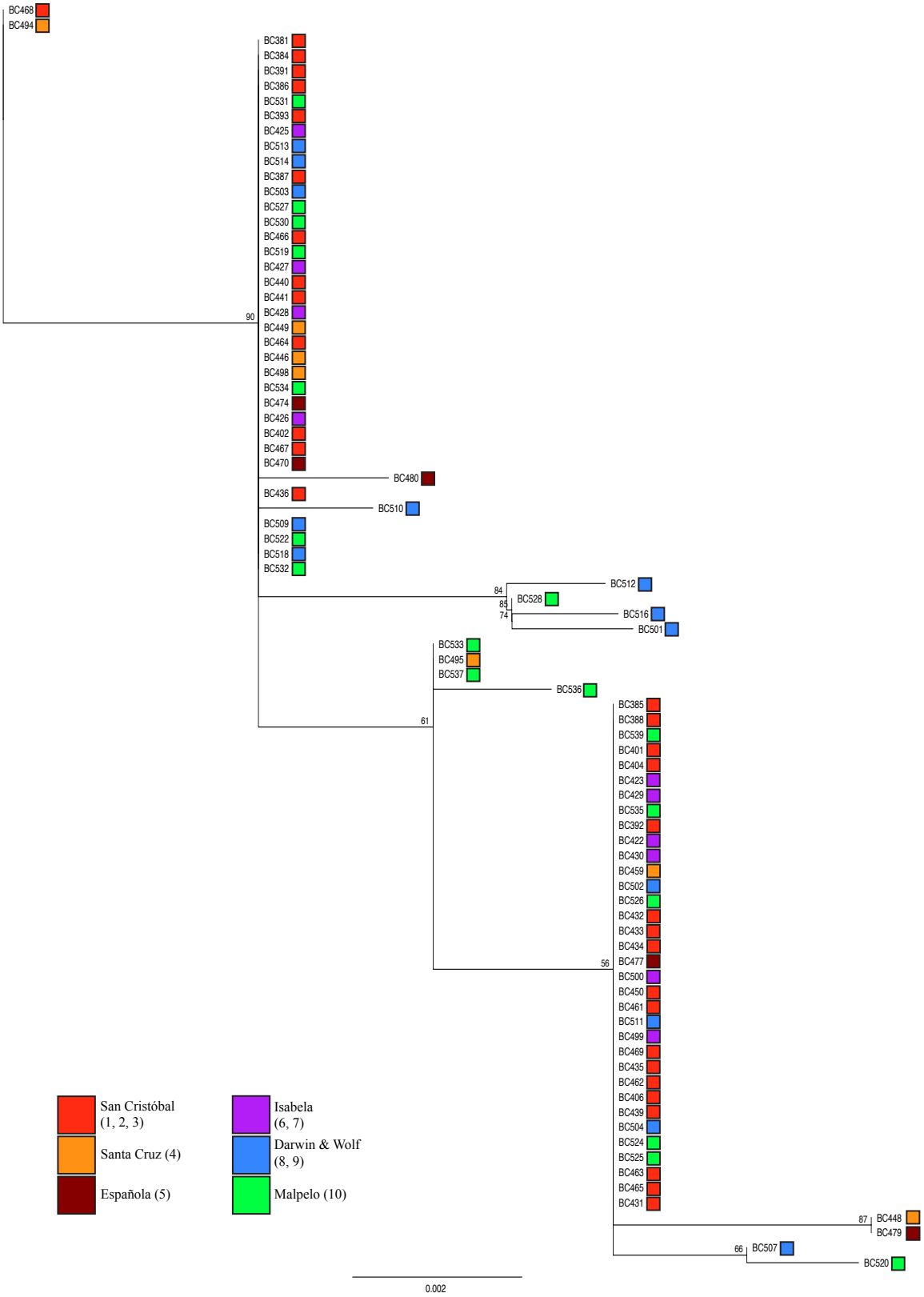
644 Fig. 2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BC431
87
BC448
BC479
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BC507
BC520
San Cristóbal (1, 2, 3)
Santa Cruz (4)
Española (5)
Isabela (6, 7)
Darwin & Wolf (8, 9)
Malpelo (10)
0.002

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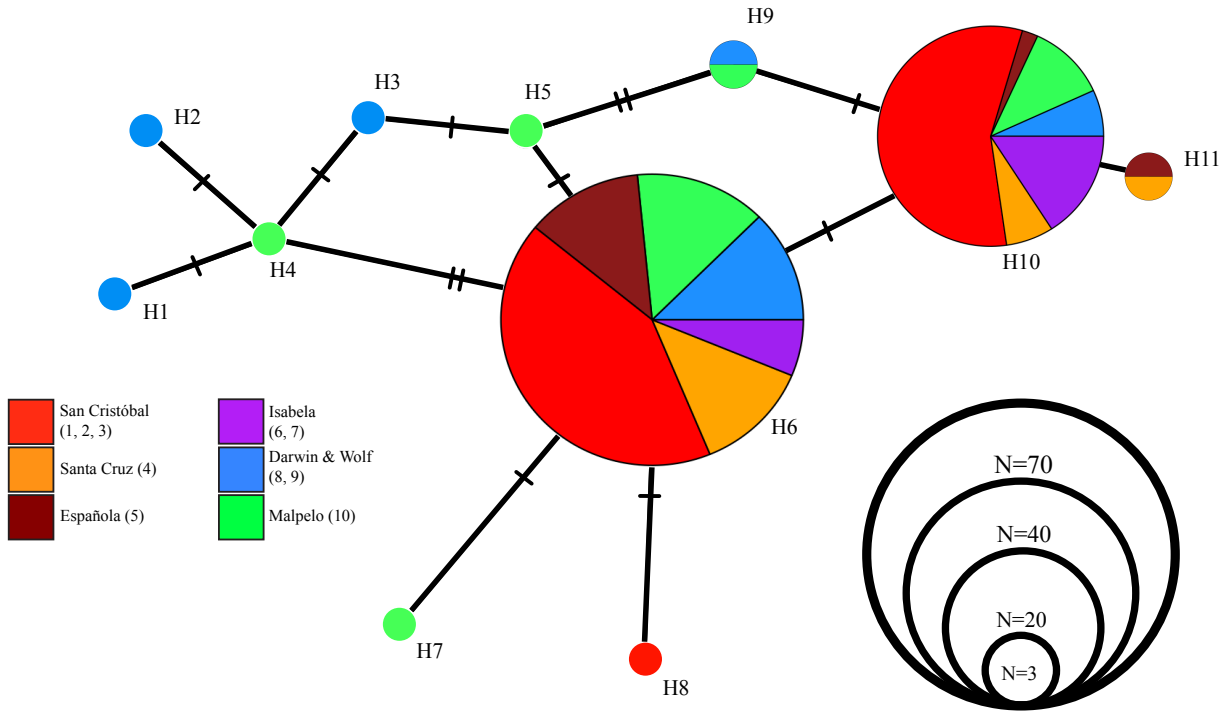
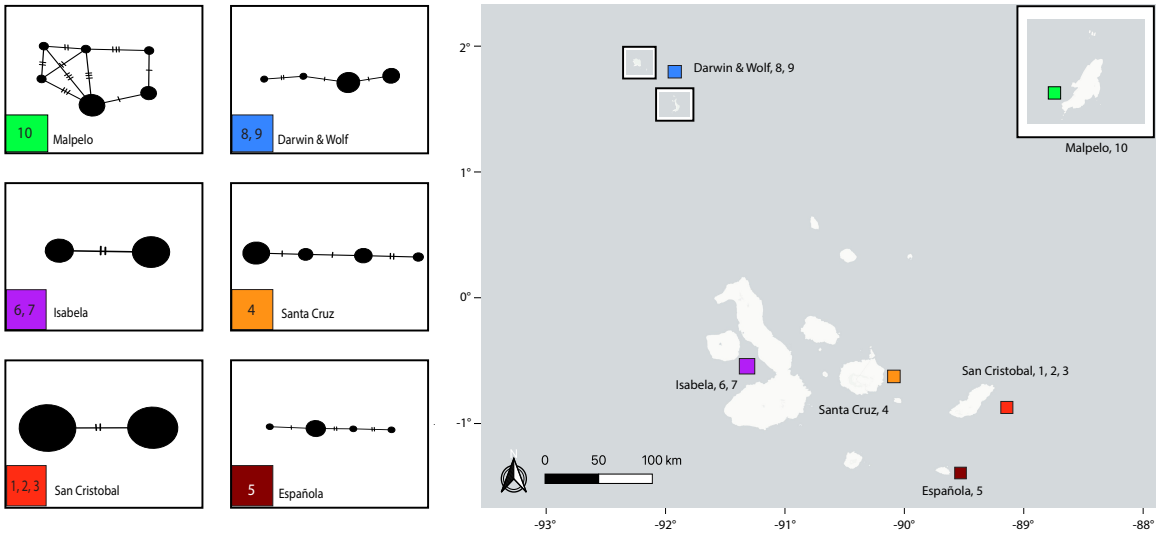
Fig. 3.1


H9
H3
H5
H2
H11
H4
H10
H1
H6
San Cristóbal (1, 2, 3)
Isabela (6, 7)
Santa Cruz (4)
Darwin & Wolf (8, 9)
Española (5)
Malpelo (10)
N=70
N=40
N=20
N=3
H7
H8

661 Fig. 3.2



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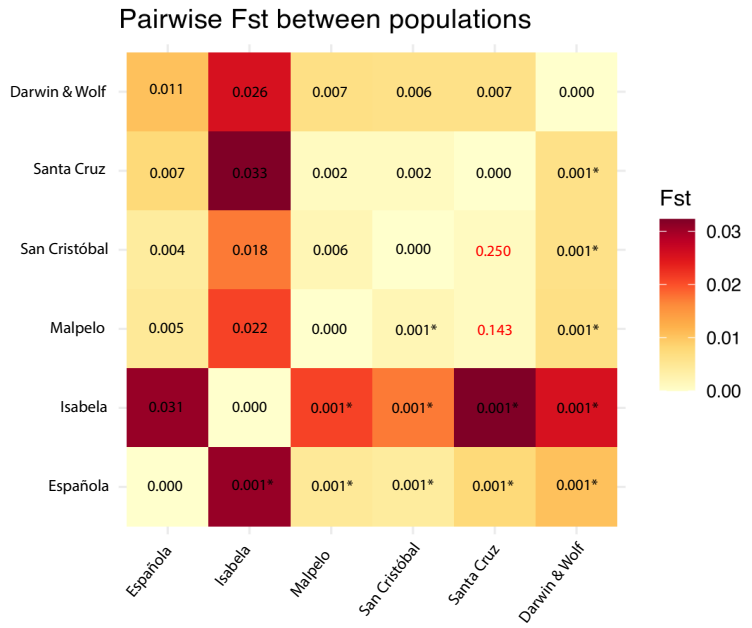
677 Fig. 4



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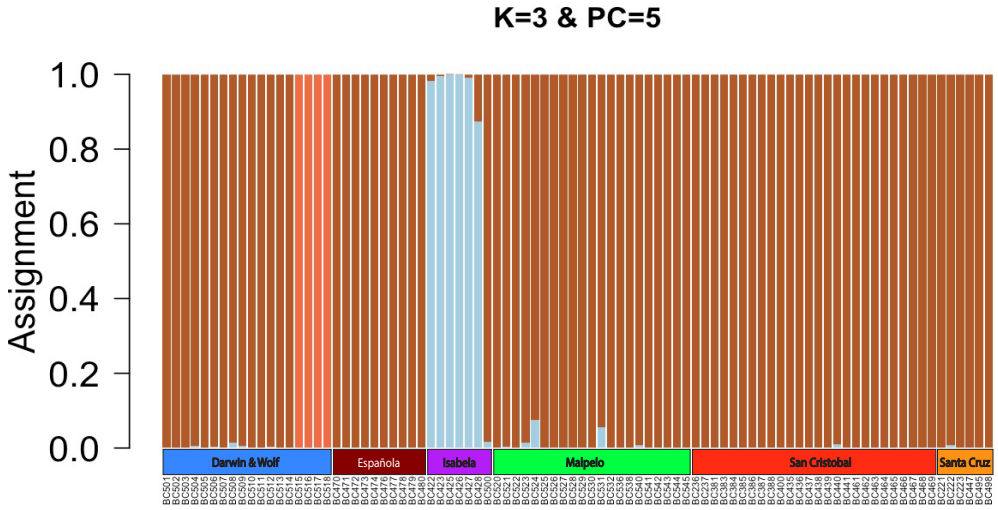
693 Fig. 5



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Supplementary Tables

Table S1. Primer sequences used for the amplification of COI and Cytochrome b genes for all 140 sequenced individuals.

Gene	Forward Primer CytBF-bac	Reverse Primer CytBR-bac	p	description
Cyt-b	5'-AATAGGAAGTATCATTCGGGTTTGATG -3'	5'-GTGACTTGAAAAACCACCGTTG -3'	7	Gene encoding subunit of the cytochrome bc1 complex (Complex III) in the mitochondrial electron transport chain.
COI	**Large Fragment HCO-2198**	**Large Fragment LCO-1490**		
	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	5'-GGTCAACAAATCATAAAGATATTGG -3'	2	Gene encoding subunit of the cytochrome c oxidase complex (Complex IV), an enzyme in the mitochondrial electron transport chain.

Table S2: Table showing the number of sampled individuals (N), allele richness (AR), observed heterozygosity (Ho), expected heterozygosity under HWE (He), adjusted He, and inbreeding (FIS) scores, for each of 6 populations. Adjusted He (*u*He) was to account for sample size differences amongst populations.

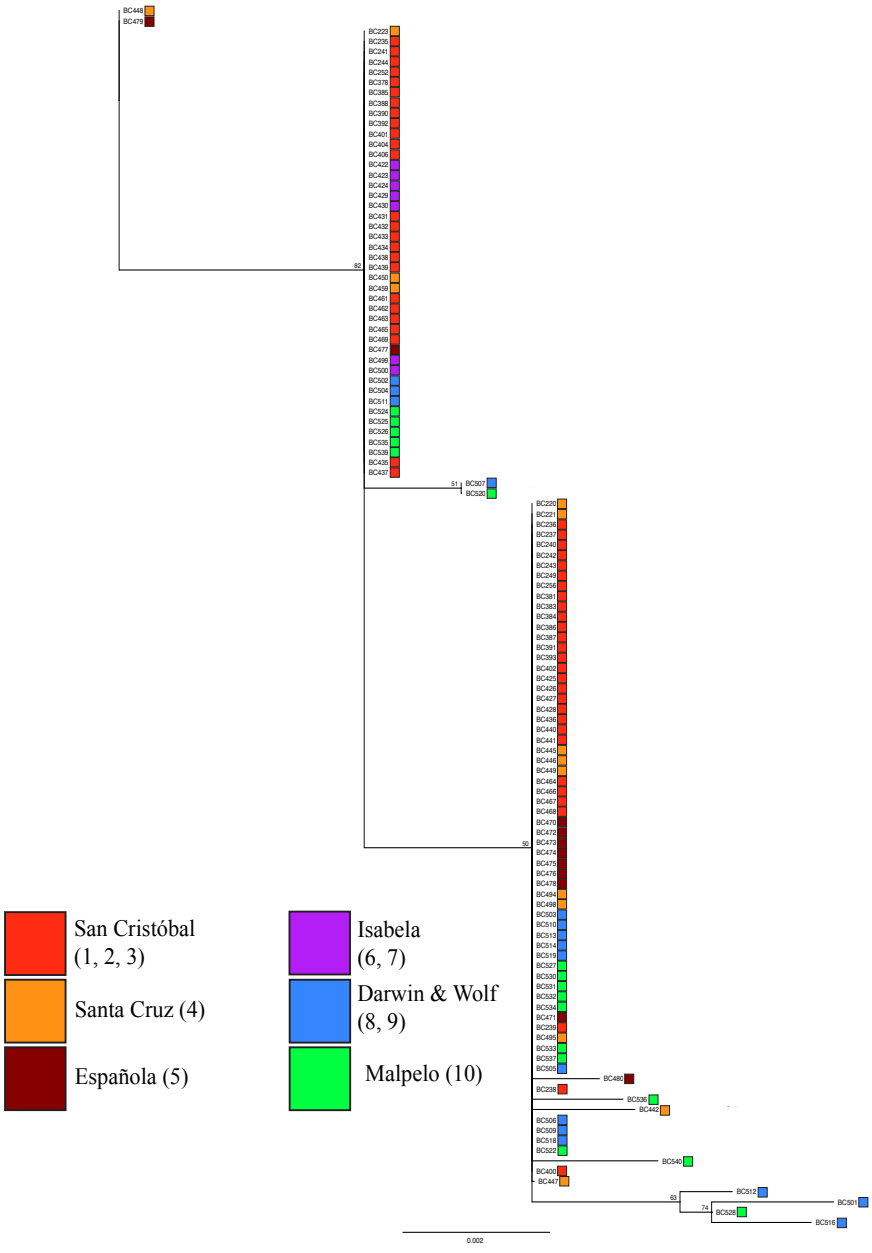
Population	N	Allele Richness (AR)	Observed Het (Ho)	Expected Het (He)	Adjusted (*u*He)	Inbreeding (FIS)
Malpelo	19	1.584	0.151	0.169	0.174	0.084
Darwin & Wolf	17	1.639	0.203	0.185	0.19	-0.057
San Cristobal	23	1.635	0.181	0.18	0.184	0.012
Española	10	1.539	0.17	0.165	0.173	-0.03
Santa Cruz	8	1.568	0.18	0.176	0.189	-0.032
Isabela	7	1.483	0.184	0.161	0.173	-0.125
Combined	84	1.574667	0.178167	0.172667	0.1805	-0.024667

Supplementary Figure Legends

Fig. S1: Complete phylogenetic tree consisting of 119 individuals. Bootstraps of 1,000 trials were used to recreate the phylogeny of *M. olfax's* Cytb gene.

Supplementary Figures

748 Fig. S1



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