Assessing common bottlenose dolphin (*Tursiops truncatus*) population structure in Mississippi Sound and coastal waters of the north central Gulf of Mexico

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ABSTRACT

1. Population structure of highly mobile marine organisms can be complex and difficult to study, but it is important to understand how populations partition themselves within their environment for accurate assessment of both natural and anthropogenic impacts and successful management. The 2010 *Deepwater Horizon* oil spill negatively impacted common bottlenose dolphins (*Tursiops truncatus*) within Mississippi Sound and the surrounding north central Gulf of Mexico (GOMx); however, little was known about their underlying population structure in these waters. Thus, it was unclear how many demographically independent populations were affected by the spill.
2. Common bottlenose dolphin samples were collected throughout inshore waters of Mississippi Sound and coastal waters of the north central GOMx. Mitochondrial DNA control region sequence data and 19 nuclear microsatellite loci were analyzed to determine how many populations are present and characterize their range throughout these waters.
3. Bayesian clustering and migration analyses identified two genetically distinct and demographically independent populations: one predominantly inhabiting Mississippi Sound and adjacent coastal waters, and a second population extending generally from offshore of Mobile Bay, Alabama east along the Florida Panhandle. Neither of these populations align with the currently delineated management stocks previously used to estimate impacts from the oil spill on common bottlenose dolphins in this portion of the GOMx.
4. These results suggest that revisions may be necessary so that management stocks accurately represent the demographically independent populations present in these

waters. Furthermore, better comprehension of underlying population structure will enhance impact assessments on common bottlenose dolphins and provide more appropriate baseline data to support future restoration and conservation objectives.

KEYWORDS: *Deepwater Horizon* oil spill, demographically independent population, management, microsatellites, mitochondrial DNA

1. INTRODUCTION

The *Deepwater Horizon* (DWH) explosion in April 2010 resulted in ~5 million barrels of oil spilling into the northern Gulf of Mexico (GOMx) over 87 days (McNutt et al., 2012). MacDonald et al. (2015) estimated that surface oil covered approximately 149,000 km^2 from west of the Mississippi River Delta in waters off Louisiana to east/northeast along the Florida Panhandle. This catastrophic event caused significant negative impacts to health and increases in both mortality and reproductive failure for marine mammals in the GOMx (DWH MMIQT, 2015; Takeshita et al., 2017).

Most of the directly quantified impacts on marine mammals in relation to the oil spill in the northern GOMx were based on studies of common bottlenose dolphins (*Tursiops truncatus*, herein referred to as "bottlenose dolphins") in inshore bay, sound, and estuarine habitats (BSEs) such as Barataria Basin in Louisiana and in Mississippi Sound. In both locations, the amount and duration of oiling was well documented (Barron, 2012; Michel et al., 2013) and numerous studies were conducted focusing on health assessment (e.g., Kellar et al., 2017; Smith et al., 2017; Barratclough et al., 2019), ranging patterns, abundance, and density estimation (McDonald

et al., 2017; Mullin et al., 2017; Wells et al., 2017), habitat preference (Hohn et al., 2017), and contaminant loads (Balmer et al., 2018; McCormack et al., 2020). The oil spill significantly impacted bottlenose dolphins in these areas, causing the death of an estimated 35% and 22% of the those inhabiting Barataria Basin and Mississippi Sound, respectively (Schwacke et al., 2014; DWH NRDA Trustees 2016; Smith et al., 2017).

To fulfill management objectives of the Marine Mammal Protection Act of 1972 (MMPA), the National Oceanic and Atmospheric Administration (NOAA) conducts research to identify and delineate "stocks" for management of marine mammals within U.S. waters. Ideally, stocks should represent single demographically independent populations (DIPs; Martien et al., 2019).

Within the U.S. GOMx, there are 36 management stocks for bottlenose dolphins, 31 of which are within BSEs. Utilizing photographic-identification (photo-ID), telemetry, and/or genetic data, population and/or community structure of bottlenose dolphins has been investigated for some of these BSE stocks, including Tampa Bay (e.g., Sellas, Wells & Rosel, 2005; Urian et al., 2009), Sarasota Bay and Charlotte Harbor/Pine Island Sound (Sellas, Wells & Rosel, 2005), St. Joseph Bay (Balmer et al., 2008), and St. Vincent Sound/Apalachicola Bay/St. George Sound (Tyson, Nowacek & Nowacek, 2011) in Florida; Matagorda Bay (Sellas, Wells & Rosel, 2005) in Texas; and Barataria Basin in Louisiana (Rosel et al., 2017; Wells et al., 2017). Although photo-ID and telemetry studies have been conducted within Mississippi Sound and surrounding waters (e.g., Hubard et al., 2004; Mackey, 2010; Sinclair, 2016; Mullin et al., 2017), there has yet to be a genetics-based population structure assessment for bottlenose dolphins occurring within the Mississippi Sound, Lake Borgne, Bay Boudreau stock (MSLBBB; Figure 1; Hayes et al., 2018).

The remaining five bottlenose dolphin stocks occur in coastal and offshore waters and cover the area between the shoreline/barrier islands and the U.S. Exclusive Economic Zone (EEZ) and include three coastal stocks (the Western, Northern, and Eastern Coastal stocks; including waters < 20 m depth), the Continental Shelf Stock (20–200 m depth), and the Oceanic Stock (> 200 m depth to U.S. EEZ; Waring et al., 2015; Waring et al., 2016). However, Vollmer & Rosel (2017) presented a comprehensive genetic study examining the number of bottlenose dolphin populations within coastal and offshore U.S. GOMx waters (from the shoreline/barrier islands to the U.S. EEZ) and provided evidence of seven genetically distinct DIPs. These seven populations do not align with at least four of the five currently delineated bottlenose dolphin stocks in these waters. The accuracy of the Northern Coastal Stock (NCS) delineation (Figure 1; Waring et al., 2016) was not well assessed in Vollmer & Rosel (2017) because few samples (n = 18) were collected within the range of this stock. Unfortunately, the NCS was also determined to have been highly impacted by the DWH oil spill; an estimated 82% of the NCS was exposed to oiling, and 38% of the stock was killed as a result of the spill (DWH NRDA Trustees 2016).

Bottlenose dolphins inhabiting inshore and coastal waters of Mississippi Sound and the north central GOMx were undoubtedly affected by the DWH oil spill; however, gaps in knowledge about the underlying population structure in these areas limit the accuracy of the population level assessment of impacts from this event. In addition, restoration plans are currently being developed to offset the impacts of the spill; it is essential to understand how many DIPs are present and better characterize their spatial range to correctly assess the benefits of restoration efforts. As part of the response to the DWH event, the Consortium for Advanced Research on Marine Mammal Health Assessment (CARMMHA) was formed. One of CARMMHA's main objectives is to fill information gaps related to the status of marine mammal

health in areas impacted by the oil, therefore efforts of this consortium have driven recent investigations focused on Mississippi Sound and the NCS. In order to meet CARMMHA's objectives and support ongoing restoration planning, the goal of this research is to identify genetic population structure of bottlenose dolphins in these areas so that the health and status of each impacted population can be accurately assessed.

2. METHODS

2.1 Sample collection, extraction, and sexing

Skin tissue samples from 614 individuals were obtained via remote biopsy (n = 576; e.g., Gorgone et al., 2008; Sinclair et al., 2015) or during capture events (n = 38; e.g., Schwacke et al., 2014), and tissue was stored frozen or in 20% DMSO/saturated NaCl. Samples were collected between 1996-2018 from all months except March and November, with 55% of samples collected in either August or September. Sampling ranged from the Mississippi River Delta east to St. Joseph Bay, FL including Mississippi Sound and waters out to the 200 m isobath (Figure 1). Outside of Mississippi Sound, samples were collected from either 1 or 2 km from the shore/barrier islands to the 200 m isobath; therefore, apart from Mississippi Sound, no samples were collected within any other BSE stocks. Genomic DNA was extracted from ~25 mg of tissue using either standard proteinase K digestion and phenol-chloroform extraction protocols (Rosel & Block, 1996) or using a DNeasy Blood and Tissue Kit (Qiagen). DNA concentration was determined through fluorometry (Hoefer DyNA Quant 200, GE Healthcare). Sex for capture samples was determined through inspection of the external genital slit, and for remote biopsy

samples through polymerase chain reaction (PCR) with ZFXY and SRY specific primers (Rosel, 2003) followed by visualization of products via 2.0–2.5% agarose gel electrophoresis.

2.2 Mitochondrial DNA sequencing

Amplification of the 5′ end of the mitochondrial DNA (mtDNA) control region using the primers L15824 and either H16265 or H16498 (Rosel, Dizon & Heyning, 1994; Rosel, Tiedemann & Walton, 1999) followed the procedures of Vollmer & Rosel (2017). PCR products were purified by either excision from a low melting point agarose gel with an overnight digestion using agarase (Sigma-Aldrich), or using ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems; ABI). Sequencing was performed in both directions using ABI Big Dye® Terminator v1.1 cycle sequencing protocols and data were collected on either an ABI 3130 or 3500 Genetic Analyzer. Four samples were processed at the University of Arizona Genetics Core where they were sequenced in both directions using ABI Big Dye® Terminator v3.1 cycle sequencing protocols and data were collected on an ABI 3730xl. All sequences were edited and the forward and reverse reads combined into a consensus sequence (Sequencher 5.4.6 Gene Codes Corporation; Geneious Prime 2020.0.5). Consensus sequences were aligned by eye (Geneious Prime 2020.0.5) and unique sequence haplotypes were identified (Geneious Prime 2020.0.5; MacClade 3.04: Maddison & Maddison, 1992). Sequence data were submitted to the GenBank database (www.ncbi.nlm.nih.gov/genbank) under accession numbers provided in Supporting Information Table 1.

2.3 Microsatellite genotyping

Samples were genotyped at 19 microsatellite loci previously optimized for *T. truncatus* (Rosel, Hansen & Hohn, 2009): Ttr04, Ttr11, Ttr19, Ttr34, Ttr48, Ttr58, Ttr63, TtrFF6 (Rosel, Forgetta & Dewar, 2005); MK5, MK6, MK8, MK9 (Krützen et al., 2001); TexVet5, TexVet7 (Rooney, Merritt & Derr, 1999); KWM12a (Hoelzel, Dahlheim & Stern, 1998); PPHO130 (Rosel et al., 1999); and EV14, EV37, EV94 (Valsecchi & Amos, 1996; see Vollmer & Rosel, 2017 for modifications applied to EV94). The reverse primer for MK8 was modified from the original Krützen et al. (2001) sequence to improve resolution of alleles in *T. truncatus*. The new reverse primer was designed as follows: 5′-GTTTCTGTGTCTCTTTGACATGCCCTCACC-3′. The reverse primers for each locus except MK6 were "PIGtailed" to reduce one base pair (bp) stutter (Brownstein, Carpten & Smith, 1996). Loci were either combined into eight multiplex PCR reactions and the PCR products further co-loaded and amplified (see methodology in Vollmer & Rosel, 2017), or combined into just four multiplex PCR reactions using the Type-it Microsatellite PCR Kit (Qiagen) as described here (Supporting Information Table 2). These latter four multiplexes were amplified in 10 µl total volume reactions containing 1x Type-it Multiplex PCR Master Mix, 0.0375–0.3 µM primer (final concentration) and 10 ng genomic DNA. Every PCR performed included both positive and negative controls, and if needed to enhance amplification, bovine serum albumin (BSA; Sigma-Aldrich) was added to the PCR reaction (final concentration 0.08–0.40 mg mL^{-1}). Genotyping was performed using an ABI 3130 or 3500 Genetic Analyzer with GeneScan™ 500 LIZ® or 600 LIZ® v2.0, respectively, as an internal size standard. Allele sizes were determined using GeneMapper (v6.0; ABI). Prior to the start of this study, approximately 70–100 bottlenose dolphin samples per Type-it multiplex were genotyped on both the ABI 3130 and 3500 in order to verify that binning and allele calls were consistent across instruments.

2.4 Quality control

For mtDNA analyses, all individuals were sequenced in both directions and manually checked for mismatches. Any indication of DNA heteroplasmy resulted in re-extraction and/or re-sequencing in at least one direction for confirmation. All unique haplotypes identified from alignments had the original sequence traces manually re-checked to verify results.

For microsatellite data, approximately 8.0% of the total data set was re-genotyped at all 19 loci. This included 21 randomly chosen samples that were re-genotyped intentionally using fresh DNA and primer dilutions, and 27 individuals that were unintentionally sampled more than once in the field and thus genotyped multiple times in the lab. In all, 48 samples were used to calculate allelic and reaction level error rates (see Vollmer & Rosel, 2017). Unintentional duplicates were identified using MSToolkit (Park, 2002) and verified based on matching mtDNA haplotype and sex. Possible null alleles, allelic dropout and potential scoring errors due to stuttering were investigated with Micro-Checker 2.2.3 (with samples separated into STRUCTURE populations (see below); Van Oosterhout et al., 2004).

2.5 Population structure analysis

To investigate genetic population structure, microsatellite data for all individuals were analyzed with the Bayesian clustering program STRUCTURE v2.3.4 without using any prior location information (Pritchard, Stephens & Donnelly, 2000). According to Wang (2017), there may be a large impact on the quality and accuracy of STRUCTURE results if sampling is unbalanced among populations. In this data set, it was not clear how many populations would be identified, and thus the influence of unbalanced sampling on analyses was unknown. However,

there was a higher proportion of samples collected in and around Mississippi Sound compared to other sampled areas (Figure 1). Therefore, there was a possibility that unbalanced sampling might affect STRUCTURE results. Following the recommendations of Wang (2017) for unbalanced sampling, the alternate ancestry prior (POPALPHAS = 1), multiple values of ALPHA that take into account the possible number of populations (K) present (ALPHA = 1/K), and both correlated and uncorrelated allele frequency models were tested. Combinations of these parameters used for analyses are shown in Table 1. For all runs, K = 1–10, 10 replicates for each K, and a burn-in of 100,000 followed by 5,000,000 iterations were used. All other parameters were left as default. To determine the best number of populations, the K estimation methods from Pritchard, Stephens & Donnelly (2000), ΔK from Evanno, Regnaut & Goudet (2005), and the parsimony estimator from Wang (2019) were compared. CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) was run using the Full Search method to obtain final membership coefficients (*Q*-values) for each individual for the best K from each estimation method. Individuals were assigned to populations using a *Q*-cutoff of 0.50. To investigate the presence of hierarchical population structure (Evanno, Regnaut & Goudet, 2005; Coulon et al., 2008), additional runs of STRUCTURE were completed on each of the populations initially identified. These runs continued until the best K = 1 (based on K estimates using the methods of Pritchard, Stephens & Donnelly, 2000 and Wang, 2019).

A second Bayesian clustering program, TESS v2.3.1 (Chen et al., 2007), that incorporates genetic and geographic information was run using the microsatellite data in combination with the collection location (latitude and longitude) for each individual. Spatial coordinates were used to compute pairwise geographic distances (great circle distance) between all individuals. The program was run assuming admixture and using the CAR model. The range of K = 2–10 was

tested, running 100 runs per K with 50,000 MCMC sweeps and a burn-in of 10,000. All other parameters remained as default. The best number of populations was chosen based on the Deviance Information Criterion (DIC) averaged over all runs for each K. The average DIC values across all Ks were graphed, and the point where the graph plateaued was taken to represent the best K. CLUMPP was run using the Greedy method to obtain final *Q*-values for each individual in the best K. Individuals were assigned to each population using a *Q*-cutoff of 0.50. Graphical outputs of results from both TESS and STRUCTURE were produced in R v3.6.0 (R Core Team, 2019) following the POPSutilities script from Jay (2011).

2.6 Characterization of diversity and differentiation

To characterize genetic diversity within the data set, standard metrics typically used in bottlenose dolphin population genetic studies were estimated as follows: the number of private alleles and probability of identity (PI) were calculated in GenAlEx v6.51b2 (Peakall & Smouse, 2006; Peakall & Smouse, 2012); the mean number of alleles, observed and expected heterozygosities per population and per locus for the microsatellite data, and the haplotype and nucleotide diversity indices for the mtDNA data were calculated in ARLEQUIN v3.5.2.2 (Excoffier & Lischer, 2010); allelic richness was calculated in FSTAT v2.9.3.2 (Goudet, 1995).

Using the mtDNA haplotype data, a median-joining network was created with NETWORK v10 (Bandelt, Forster & Röhl, 1999; Fluxus Technology, 2020). Furthermore, two ecotypes of bottlenose dolphins, offshore and coastal, are found in the GOMx (Vollmer & Rosel, 2013). Ecotype can be inferred based on where an individual's mtDNA haplotype is located in a Bayesian tree. To identify whether samples were of the coastal or offshore ecotype, a Bayesian phylogenetic analysis using the control region alignment was performed using MrBayes v3.2.7a

(Ronquist et al., 2012). Two Atlantic spotted dolphin (*Stenella frontalis*) sequences were used as outgroups and the program jModeltest 2.1.10 (Guindon & Gascuel, 2003; Darriba et al., 2012) was used to determine the most appropriate model of nucleotide substitution to use for the phylogenetic analysis (HKY+I+G). In MrBayes, two independent analyses of four chains were run, sampling trees every 1,000 generations with the first 25% of trees discarded as burn-in. Twenty-five million generations were run and convergence was verified by examining the average standard deviation of split frequencies, stability of the log likelihood values of the cold chains, and with the program TRACER v1.7.1 (Rambaut et al., 2018). For all samples, ecotype was inferred based on where individuals were located in the Bayesian tree.

To investigate the level of differentiation among the populations identified in the clustering analyses, analyses of molecular variance (AMOVAs) were run in ARLEQUIN to estimate global and pairwise F_{ST} and Φ_{ST} with the nuclear and mtDNA data. Based on results from jModeltest, the Tamura & Nei (1993) model was used with Gamma $a = 0.02$ to estimate Φ_{ST} for the mtDNA data. GENEPOP v4.7.2 (Rousset, 2008) was run using the microsatellite data from each STRUCTURE population to investigate the presence of linkage disequilibrium (LD) and any departures from Hardy-Weinberg equilibrium (HWE). The potential of sex-biased dispersal was investigated with the mtDNA data using ARLEQUIN and with microsatellite data using FSTAT.

STRUCTURE identified two populations (see Results) of very different sample size. Therefore, to account for bias arising from the large disparity in size in estimates of genetic differentiation, diversity, and sex-biased dispersal, subsets of the larger population were created to perform comparisons with the smaller population. Specifically, 10 subsets of the mtDNA and microsatellite data were created to represent the larger population by randomly assigning 42

individuals (the number of individuals assigned to the smaller population) from the larger population identified by STRUCTURE. The entire larger population as well as these subsets were each analyzed along with all samples assigned to the smaller population.

2.7 Analyses of migration, selection, and relatedness

Using the microsatellite data, migration rates between STRUCTURE populations were estimated in MIGRATE v4.4.4 using the Brownian motion mutational model and Bayesian inference (Beerli, 2006; Beerli, 2009; Beerli & Palczewski, 2010). To account for uneven population sizes, random subsets containing the genotypes from 42 individuals were created in the program to represent each population in analyses (Beerli P, 2020, personal communication). Slice sampling was used to generate posterior distributions, and three uniform prior distributions for both the mutation scaled immigration rate (M) and theta (Θ) were tested with minimum, maximum, and delta parameters as follows: 1) 0, 50, 5; 2) 0, 100, 10; and 3) 0, 200, 20. Additional settings included 1 long chain with 100,000 recorded steps, 100 long increments, a burn-in of 10,000, a static heating scheme with temperatures 1, 1.5, 3 and 1,000,000, and for each run 50 replicates were performed. These additional settings were established after a series of preliminary runs and recommendations from the program's author (Beerli P, 2020, personal communication). For each of the three sets of prior distributions, two migration models were tested and compared to one another: 1) one that treated each STRUCTURE population separately and utilized a full migration matrix to estimate parameters between all populations, and 2) a panmictic model that analyzed all data as if it were from a single population. To determine the best model, the log Bayes Factor was calculated with the package mtraceR (Pacioni et al., 2015) run in R using the overall Bezier approximation score for each model tested.

BAYESCAN v2.1 (Foll & Gaggiotti, 2008) was used to detect the presence of any nuclear loci under selection. All default parameters were applied except the thinning interval was set to 500 and 1,000 pilot runs were completed. To investigate the presence of related individuals in each STRUCTURE population, the R package *related* (Pew et al., 2015) was used. For this analysis, first 42, 100, and 250 pairs were simulated for each level of relatedness for each population. Relatedness was then estimated with the simulated data sets using two likelihood estimators (dyadml: Milligan, 2003; trioml: Wang, 2007) and four moment estimators (lynchli: Lynch, 1988; Li, Weeks & Chakravarti, 1993; lynchrd: Lynch & Ritland, 1999; quellergt: Queller & Goodnight, 1989; wang; Wang, 2002). The Pearson correlation coefficient was used to compare expected and observed relatedness values for each estimator to determine the estimator that correlated best with the simulated data. The best estimator was then used to determine relatedness (r) among individuals in the real data from the STRUCTURE populations. A pair of individuals was considered highly related if $r \geq 0.50$. The inclusion of loci under selection and/or highly related individuals can bias estimates of genetic diversity (e.g., Futuyma, 1986; Excoffier, Hofer & Foll, 2009; Wang, 2018). Therefore, all loci identified as potentially being under selection as well as any highly related individuals were removed and all analyses were repeated.

3. RESULTS

Of the 614 samples, 31 were determined to be duplicates and removed from the data set (some individuals were sampled more than two times). The remaining 583 samples comprised 230 females and 353 males. A 354 bp fragment of the mtDNA control region was sequenced for all samples, and 51 haplotypes were identified, including 25 that contained single base

heteroplasmy (Vollmer et al., 2011; Supporting Information Table 3). All samples with a heteroplasmic haplotype (n = 35 individuals) were excluded from analyses conducted with mtDNA data except for the phylogenetic analysis performed to determine ecotype. Microsatellite genotypes were successfully determined for all samples at all loci except for one sample whose genotype could not be determined for Ttr63. For all samples, ecotype, haplotype, GenBank accession numbers, sex, and microsatellite genotype data can be found in Supporting Information Table 4.

No discrepancies were found upon re-genotyping ~8.0% of the entire data set, resulting in an overall allelic- and reaction-level error rate of 0.0%. Analyses of all samples, as well as the subsets created from individuals of the larger population (see below), revealed no consistent occurrence across populations (or subsets) for the presence of null alleles, allelic dropout, or scoring error.

3.1 Population structure

Results from STRUCTURE supported the best K = 2, identifying a "green" population (n = 541) and "blue" population (n = 42; Figures 2 and 3a). This result was consistent for all tested parameters and across the three K estimation methods with the exception of Wang's K for the uncorrelated allele frequency model (FREQSCORR = 0; Table 1, Supporting Information Table 5). Across the different parameters tested, the individuals assigned to each of the two populations were identical, again except for when K = 2 using the uncorrelated allele frequency model where fewer individuals were assigned to the blue population. Re-running each population separately did not identify any further hierarchical population structure.

TESS similarly supported that the best K = 2, and compared to STRUCTURE identified a similar green population (n = 550) and a blue population (n = 33; Figure 3b). There was no obvious plateau in the graph of average DIC per K value, but the amount of change in average DIC per run was greatest between K = 2 and 3 (Supporting Information Figure 1a). Furthermore, individual assignments were examined for K = 2 thru 5 and, regardless of K, individuals were never assigned to anything other than two populations (e.g., when K = 3 only two populations contained individuals with $Q \geq 0.50$, the highest Q-value in the third population was 0.28, therefore no individuals were assigned to the third population). Individuals assigned to the two populations were not identical across different Ks, but were very similar and overall corresponded very closely to the individual assignments from STRUCTURE K = 2 results (Figure 3).

3.2 Genetic diversity

Private microsatellite alleles and mtDNA haplotypes were identified in both populations and in all subsets, although a majority of the private haplotypes (especially for the green population and subsets) were found only once (Supporting Information Table 3 and 6). The probability that any two individuals randomly drawn from the green or blue population would have the same genotype was very low with PI = 8.8×10^{-17} and 3.9×10^{-19}, respectively. The mean observed heterozygosity across all microsatellite loci was 0.6199 (± 0.1994) for the green and 0.7005 (± 0.1527) for the blue population. For the mtDNA data, haplotype diversity was 0.7816 (± 0.0080) and 0.8943 (± 0.0203), and nucleotide diversity was 0.0053 (± 0.0034) and 0.0202 (± 0.0107) for the green and blue populations, respectively. Additional diversity indices, mean allele number, and mean heterozygosity values for each population and all subsets are

provided in Supporting Information Table 6, and results for allelic richness, mean number of alleles, and heterozygosity by locus in each population and subset are given in Supporting Information Table 7. Overall, these diversity results are comparable to those found for other bottlenose dolphin populations within the GOMx (Sellas, Wells & Rosel, 2005; Litz et al., 2012; Vollmer & Rosel, 2017), the western North Atlantic (Rosel, Hansen & Hohn, 2009; Litz et al., 2012), the eastern North Atlantic (Nykänen et al., 2019), and Hawaii (Martien et al., 2012).

Bayesian phylogenetic analysis of the 51 mtDNA haplotypes resulted in two well-supported clades (posterior probability = 1.00) clearly separating coastal (n = 46) and offshore (n = 5) ecotypes (Supporting Information Figure 2). In total, 555 individuals exhibited haplotypes corresponding to the coastal ecotype and 28 corresponding to the offshore ecotype (Supporting Information Table 3). Relationships among non-heteroplasmic haplotypes and relative haplotype frequencies are represented in a median-joining network shown in Figure 4. This network further depicts the large separation between coastal and offshore ecotypes (separated by nine mutational steps).

3.3 Genetic differentiation

Significant genetic differentiation between the two populations was detected with an F_{ST} = 0.05 for the microsatellite data, and F_{ST} = 0.16 and Φ_{ST} = 0.65 for the mtDNA data (all $p <$ 0.0001). In analyses comparing the green population subsets with the blue population, results were comparable to those above with F_{ST} ranging from 0.04 – 0.05 and 0.13 – 0.21 for microsatellite and mtDNA, respectively, and Φ_{ST} ranged from 0.36 – 0.51 (all $p < 0.0001$; Supporting Information Table 6). No significant departures from HWE or evidence of LD were detected within or across any populations.

When comparing the entire green versus the entire blue population, no tests for sex-biased dispersal using microsatellite data were statistically significant (all $p > 0.05$), and overall F_{ST} and Φ_{ST} estimates based on the mtDNA data were comparable between sexes (females: $F_{ST} = 0.19$, $\Phi_{ST} = 0.73$, $p < 0.0001$; males: $F_{ST} = 0.14$, $\Phi_{ST} = 0.70$, $p < 0.0001$) suggesting limited dispersal for both sexes. In comparisons using the green population subsets, results based on mtDNA data were similar as above (Supporting Information Table 8); however, with the microsatellite data, comparisons with some subsets did produce significant metrics although this did not occur consistently across subset comparisons (Supporting Information Table 9).

The migration model of two separate populations was ranked first over panmixia regardless of the priors used (Table 2, Supporting Information Table 10). Results based on an M and Θ prior distribution with minimum = 0, maximum = 50, and delta = 5 are presented here. Estimates of migration were quite low between the two populations with 0.01 (2.5% and 97.5% percentiles = 0.00, 0.17) immigrants per generation ($N_e m$) in the green population arriving from the blue, and 0.14 (0.00, 0.47) immigrants per generation in the blue population arriving from the green. Results using other prior distribution values provided very similar results with all $N_e m < 1.0$ (Supporting Information Table 10).

Three microsatellite loci (EV17, EV37 and Ttr63) were detected by BAYESCAN as being potentially under selection (Supporting Information Figure 3). For relatedness analyses, the dyadml estimator correlated best with all simulations of the blue population, and the trioml estimator was the only one that ranked either first or second best across all green population simulations. Using the dyadml estimator with the blue population and the trioml estimator with the green population to determine *r*-values, a relatively high number of related pairs ($r \geq 0.5$) was identified within the green population (n = 174) compared to the blue (n = 3). Based on

relatedness and selection results, all analyses were re-run after removing the three microsatellite loci listed above, plus 94 and 2 potentially related individuals from the green and blue populations, respectively. Results from re-analyses with STRUCTURE and TESS did not change greatly (i.e., the same two populations were identified with similar individual assignments; Supporting Information Figures 1, 4, and 5), and all statistical analyses of differentiation remained significant (data not shown) with values very similar to those presented for the entire data set.

4. DISCUSSION

Genetic evidence supports the presence of two differentiated common bottlenose dolphin populations inhabiting coastal waters from the Mississippi River Delta east to St. Joseph Bay, FL and including the inshore waters of Mississippi Sound (Figures 3 and 5). Specifically, a population (green) was identified that is largely found west of Mobile Bay encompassing inshore and coastal waters in and around Mississippi and Chandeleur sounds, and a second population (blue), present in coastal waters east of Mobile Bay to approximately Mexico Beach, FL. The green population also is present in waters < 20 m depth west of St. Joseph Bay, as identified by eight samples collected along the Florida Panhandle within the 20 m isobath that had an average STRUCTURE $Q = 0.95$ (Figure 3). Connection of the green population between the waters of St. Joseph Bay and Mississippi Sound is also suggested from telemetry and/or photo-ID data for three dolphins: one with confirmed sightings near St. Joseph Bay and also 100 km west off Destin, FL; and two others both with confirmed sightings within St. Joseph Bay and again both just west of the MS/AL state border off Petit Bois Island, MS (Balmer et al., 2016).

The two populations differ in preferred water depth with the green population occupying shallow and nearshore waters of Mississippi and Chandeleur sounds and extending past the barrier islands of Mississippi Sound to around the 40 m isobath. A few individuals assigned to this green population were sampled beyond the 40 m isobath, close to the 100 m isobath (n = 14 sampled in waters between 40–100 m depth south of Mississippi Sound with average STRUCTURE $Q = 0.97$). In contrast, individuals assigned to the blue population were not sampled within the shallow waters of Mississippi and Chandeleur sounds and were mostly found in coastal waters between the 20 m and 60 m isobaths.

4.1 Demographic independence of the populations

Levels of differentiation between the two populations are similar to those found among some of the seven coastal and offshore GOMx populations identified in Vollmer & Rosel (2017), and other currently recognized stocks in BSE areas of the GOMx (Sellas, Wells & Rosel, 2005; Rosel et al., 2017). Furthermore, estimates of migration were quite low, with < 1 individual migrating between these populations per generation. Hastings (1993) estimated that populations exchanging less than 10% of individuals from each population each year could maintain demographic independence. Additionally, after testing several models of dispersal for marine mammals, Taylor (1997) determined that dispersal rates of less than a few percent per year between a population that is harvested and one that is not are not enough to compensate for removal from the harvested population. Thus, Taylor (1997) concluded that when dispersal is less than a few percent per year, two populations should be managed separately to meet the MMPA goal to maintain them as functioning elements of their ecosystem. Using generalized boundaries for each population, an abundance of 7,335 (95% CI: 2,219–24,235; SE = 4,468) for the blue population and 9,522 (95% CI: 6,119–14,818; SE = 2,116) for the green population was

estimated using survey data collected in summer 2011 (see Supporting Information on Abundance Estimation). Considering Hastings (1993), exchange of 10% between each population per year would equate to 733 individuals from the blue population and 952 from the green population. Calculated estimates of migration in this study are much less than this, at < 1 individual *per generation* (considering a generation time of 21 years for bottlenose dolphins; Taylor et al., 2007), thus well below the thresholds of Hastings (1993) and Taylor (1997). Taken together, estimates of genetic differentiation and low levels of migration indicate that internal factors (i.e., births and deaths within the population) are more important to population dynamics than external factors (i.e., immigration and emigration between populations) and support the designation for both populations as DIPs. Furthermore, if these DIPs are not managed as separate stocks and one is depleted due to some stressor (significant reduction in food source, human caused mortality, etc.), immigration from the adjacent DIP would not be expected to be sufficient to prevent a decline in abundance or even extirpation of the declining population.

4.2 Comparisons to current stock boundaries

This study was able to increase the number of individuals analyzed (from 18 to 164) from within the boundaries of the NCS compared to Vollmer & Rosel (2017). Genetic data support that dolphins within Chandeleur Sound, which are currently within the delineation of the NCS, are actually part of the same population as those within and around Mississippi Sound. Although the full extent of the NCS in the east was not covered in this current study (the limit of the current data set was further west around 85.4°W than the eastern NCS boundary at 84.0°W), data support that the current boundaries of this stock do not align well with either of the populations identified here. For the MSLBBB, the few dolphins sampled within Bay Boudreau did assign to

the same population as those in and around Mississippi Sound, however no samples were available from within Lake Borgne, therefore we were not able to confirm the population status of the latter. Overall for both the green and blue populations, it is possible their ranges extend even further into adjacent waters in which samples were not analyzed for this study. Nonetheless, with the majority of individuals in both the blue and green populations extending at least out to the 40–60 m isobaths, they do not align spatially with current delineations for either the NCS or MSLBBB. Therefore, to better fulfill the management objectives of the MMPA, current stock boundaries should be revised to more accurately represent the DIPs present in these waters.

4.3 Characterization of populations in relation to the DWH oil spill

During the DWH event, surface oil from the spill overlapped with almost the entire geographic area of this study (Figure 5). Research conducted shortly after the oil spill utilized the current stock delineations, and abundance estimates based on those delineations, for impact assessment. As previously mentioned, two of the most heavily impacted bottlenose dolphin stocks were determined to be the MSLBBB and NCS. Here, we consider the overlap between the oil spill footprint and the ranges of the two populations identified in this study. Before making this comparison, it was confirmed that the samples within the blue and green populations accurately represent each population's range both prior to and after the oil spill by 1) mapping locations of samples collected before and after the spill (Supporting Information Figure 6), and 2) by performing additional STRUCTURE runs on all samples collected prior to the DWH oil spill, and separately on those collected after the oil spill (data not shown). Results revealed the presence of the same blue and green populations suggesting that population structure did not change in relation to the oil spill.

The green population extends beyond the barrier islands of Mississippi Sound onto the continental shelf east of Louisiana and south of Mississippi. Given the large geographic range of this population, a significant proportion of it, and particularly individuals inhabiting the waters beyond the barrier islands, overlapped with the spill footprint, suggesting that more individuals from this population were exposed to oil than previously thought. Furthermore, because this population extends further offshore and closer to the wellhead, a portion of it may have been exposed to a higher level of oiling over the course of the spill compared to waters closer to shore (Figure 5). Considering only the samples included in this study, the green population was likely exposed to over three times more oil on average when compared to individuals sampled within the MSLBBB as currently delineated (Table 3). Health assessments focused on bottlenose dolphins within Mississippi Sound were conducted under the assumption that those animals were part of a population that primarily encompassed a much smaller geographic area (i.e., Mississippi Sound, Lake Borgne, and Bay Boudreau). Therefore, when various types of injury were quantified, it is possible that, given the actual extent of the population inhabiting (in part) Mississippi Sound, the greater overlap with the oil footprint, and exposure to higher levels of oil, overall health impacts for this population were underestimated.

Although a much smaller number of individuals were assigned to the blue population, the geographic distribution includes waters that had a higher amount of surface oil over the course of the spill compared to waters within the 20 m isobath (Figure 5). When comparing individuals assigned to the blue population versus those sampled within the NCS, the blue population was exposed to larger amounts of oil (Table 3). It was estimated that 38% of the bottlenose dolphins within the NCS died due to the oil spill, one of the highest estimates of all BSE and coastal stocks examined (DWH NRDA Trustees 2016). It is possible this proportional estimate should

be even higher if the actual range of the blue population, and not just the delineation of the NCS, is taken into account.

Accurate estimates of population abundance and a comprehensive characterization of population ranges will be important to monitor recovery from DWH oil spill impacts and implement effective restoration planning for bottlenose dolphin populations in the GOMx. Results from this study highlight that a critical component for understanding impacts is an understanding of the underlying population structure. Information from inshore and coastal bottlenose dolphins, which are more tractable to study, provide the foundation to model impacts for cetacean species that are more difficult to assess in offshore environments. Therefore, linking health data with accurate information on source populations for dolphins sampled in Mississippi Sound or coastal areas of the north central GOMx has important implications for broader injury assessment and the restoration planning and monitoring that follow.

4.4 Biogeographic characterization of the populations

Mississippi Sound is a shallow (average depth at mean low water ~3.0 m; Eleuterius, 1978; Kjerfve, 1986) body of water that experiences large fluctuations in both sea surface temperature (location means range from ~9–17°C in winter and from ~26–33°C in summer; Christmas, 1973) and salinity. The latter can range from < 10 to 30 ppt varying not only seasonally but also spatially within the same season (Eleuterius, 1976; Eleuterius, 1977). Compounding the irregularity and intensity of these fluctuations are impacts from both natural and managed freshwater input from the Mississippi, Pearl, Pascagoula, and Mobile rivers (Eleuterius, 1977; Kjerfve, 1986; Orlando et al., 1993), and the relatively frequent occurrence of

intense weather systems (e.g., 59 named hurricanes and tropical storms have struck the north central GOMx between 1950 and 2018; NOAA Historical Hurricanes Track v.4.0, 2019).

Despite the sometimes-challenging environmental conditions, bottlenose dolphins have developed strategies allowing them to thrive within Mississippi Sound; bottlenose dolphins with high site-fidelity have been well documented (Hubard et al., 2004; Mackey, 2010; Sinclair, 2016; Mullin et al., 2017). Compared to within the sound, the physical habitat outside the barrier islands, particularly out to the 40 m isobath, is similar, i.e., the shelf is relatively wide with low relief and a gradual slope (Davis, 2017). The numerous large passes and dredged channels connecting the sound with adjacent coastal waters of the continental shelf allow high connectivity between the sound and nearshore coastal habitat. Therefore, it is perhaps not surprising that the green population is not restricted to the inshore waters within the barrier islands, as the adjacent coastal habitat is not dramatically unlike that of Mississippi Sound. It is possible that some individuals within the green population utilize different parts of the overall range seasonally and/or when conditions in certain areas become unfavorable (e.g., extreme lows for salinity and/or temperature) over relatively short periods of time. Unfortunately, the distribution of samples (for either population) collected per season was not distributed well enough to examine seasonal differences (Supporting Information Table 11). However, previous work has found evidence for seasonal movements and suggested that some bottlenose dolphins, at least temporarily, may reside in coastal waters outside of Mississippi Sound (Lohoefener et al., 1987; Hubard et al., 2004; Miller et al., 2013; Mullin et al., 2017) supporting the genetic results presented here. Future work could combine both photo-ID and genetic data to map confirmed sightings of genetically sampled individuals to further investigate distribution patterns and potential seasonal movements.

Compared to waters south of Mississippi, the bathymetry underlying the range of the blue population in waters south of Alabama and particularly off the Florida Panhandle is quite different. Here, the continental shelf narrows to ~25 km wide and features a relatively steep slope (6 m km^{-1}; Hines & Locker, 2011). Along the panhandle, the distance between either 1 or 2 km from shore and the 20 m isobath (the current northern and southern delineation of the NCS, respectively) narrows significantly to a minimum of ~1 km off Destin, FL and a maximum of 15 km wide off Cape San Blas, FL. There are no obvious habitat characteristics that might act to restrict the occurrence of a bottlenose dolphin population to only occur within this relatively small area. Furthermore, these north central GOMx coastal waters are regularly influenced by eddies, currents, and upwelling and/or downwelling events often associated with the DeSoto Canyon and are also characterized by complex shelf/slope water transport and exchange (e.g., Hamilton & Lee, 2005; Hamilton et al., 2015; Weisberg, Zheng & Liu, 2016). On occasion warm oceanic water can even intrude within several kilometers of the Florida Panhandle coast bringing oceanographic characteristics more common in deeper oceanic waters closer to shore (Huh, Wiseman & Rouse, 1981). It is therefore not very surprising that for the individuals assigned to the blue population almost half (45%) are of the offshore ecotype, compared to only 2% of the individuals assigned to the green population. Vollmer & Rosel (2017) found that the two coastal populations ranging predominantly between the 20 and 200 m isobaths in the GOMx also contained a mix of both coastal and offshore ecotypes. In fact, one of these populations, the East Outer Shelf, aligns well geographically with the blue population from the current study. However, additional sampling and larger-scale (geographically) analyses are necessary to further investigate the range and composition of the blue population, as well as the presence of any other populations within these waters of the GOMx.

Similar to the findings of Vollmer & Rosel (2017), a biogeographic break between bottlenose dolphin populations is apparent in coastal waters south of Mobile Bay, AL (Figures 3 and 5). Although some overlap occurs between the two populations, the blue population appears to have its western-most extent here, and fewer individuals assigned to the green population are found east of the Mobile Bay area. Additional support for a biogeographic break off Mobile Bay also comes from studies on Atlantic spotted dolphins (*Stenella frontalis*; Viricel & Rosel, 2014), and other fish and shrimp species (e.g., McClure & Greenbaum, 1999; Portnoy & Gold, 2012; Drymon et al., 2020).

The identification of the two populations in this study continues to support previous research showing that bottlenose dolphins can exhibit complex population structure. It is important to consider how some species, particularly those with complex social intelligence like common bottlenose dolphins (Wells, 2003), partition themselves in one region may not be the same as in other regions, even if many of the habitats within each region appear generally similar. For example, bottlenose dolphins inhabiting Barataria Basin, LA have been found to exhibit significant fine-scale structure with at least two genetically differentiated populations occurring within this comparatively small BSE (1673 km^2; USEPA, 1999; Rosel et al., 2017; Wells et al., 2017). Furthermore, bottlenose dolphins within Barataria Basin are genetically differentiated from those inhabiting adjacent coastal waters outside of the bay (Rosel et al., 2017). On the other hand, within Mississippi Sound (2129 km^2; Eleuterius, 1978), there is no genetic evidence suggesting the presence of multiple populations, nor do data support differentiation between bottlenose dolphins within the sound and those in adjacent nearshore coastal waters. Common amongst many environments is that population dynamics are likely influenced directly and/or indirectly by complex interactions with both natural and

anthropogenic elements, such as oceanographic features, freshwater intrusion, and the many ways humans utilize the marine environment.

4.5 Conclusion

Overall this research provides improved knowledge of the DIPs in Mississippi Sound and the north central GOMx and how they may be influenced by various biogeographic factors. Moving forward, data presented here support that stock boundaries should be revised to fulfill the objectives of the MMPA and ensure successful conservation of these populations. A better understanding of population ranges and revised stock delineations will inform future abundance estimates and allow for a more accurate assessment of impacts on bottlenose dolphins, as well as enhance the development of statistical models for restoration planning for marine mammals beyond the waters of the north central GOMx. Finally, additional data from telemetry, photo-ID and genetics are needed to investigate the extent of the populations identified here and the presence of any seasonal movements, and to better understand the relationships with populations in adjacent waters not examined in this study. Taken together, a better understanding of underlying population structure and the forces influencing population dynamics is critical for not only successful management and conservation of bottlenose dolphins, but also when evaluating population-level impacts, planning restoration projects, and performing future assessments.

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TABLE 1 Parameter settings used in STRUCTURE following the recommendations of Wang (2017) that take into account the possible number of populations (K) present (ALPHA = 1/K), and both correlated (FREQSCORR = 1) and uncorrelated allele frequency (FREQSCORR = 0) models. The alternate ancestry prior (POPALPHAS = 1) was used for all runs. Best K estimates using the Pritchard, Stephens & Donnelly (2000), Evanno, Regnaut & Goudet (2005) and Wang (2019) methods are given.

ALPHA	FREQSCORR	Pritchard K	Evanno ΔK	Wang K
0.25	1	2	2	2
0.33	1	2	2	2
0.50	1	2	2	2
0.50	0	2	2	1

TABLE 2 Comparison of models run in MIGRATE using the mutation scaled immigration rate (M) and theta (Θ) prior distributions of minimum, maximum, delta = 0, 50 and 5, respectively. Log(mL): Log marginal likelihood (Bezier approximation score); LBF: log Bayes Factor.

Model	Log(mL)	LBF	Model Rank	Model Probability
2 Populations	-2878090.15	0	1	1.00
Panmixia	-6839855.43	-7923531.00	2	0.00

TABLE 3 Calculated cumulative surface oil coverage in cubic meters (m^3) for samples grouped based on assignment to a current NOAA-delineated management stock or either the green or blue populations from this study. For some samples an oil coverage value was not available, and 60

samples were collected within the delineation of the Continental Shelf Stock and therefore were not included in either of the first two rows.

Samples grouped by	**Total sample size**	**# Samples with measured value**	**Average oil coverage value (m^3)**
Mississippi Sound, Lake Borgne, Bay Boudreau Stock	359	342	59.7
Northern Coastal Stock	164	157	223.2
Green Population	541	518	192.5
Blue Population	42	40	375.6

FIGURE 1 Map of all bottlenose dolphin samples (black circles) collected for this study within the north central Gulf of Mexico. The generalized area of the Mississippi Sound, Lake Borgne, Bay Boudreau Stock (red dashed line) and the Northern Coastal Stock (purple dotted line) are outlined. Note the eastern boundary for the Northern Coastal Stock delineation is 84.0°W and not shown on this map. The 20 and 200 m isobaths are shown.

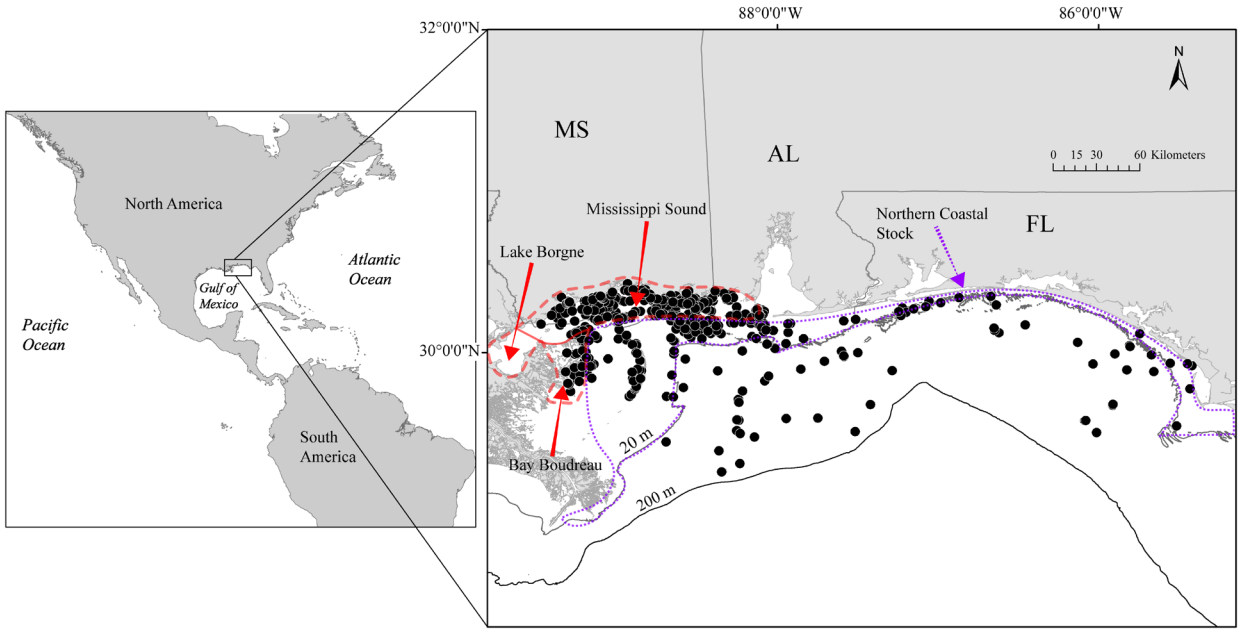
FIGURE 2 Bar plot of K = 2 from the STRUCTURE run with ALPHA = 0.50 and FREQSCORR = 1. Each vertical bar on the *x*-axis represents a single individual and is shaded based on the proportion (*Q*-value) assigned to each population. Towards the right end of the plot a black line separates individuals assigned to the green (n = 541) and blue populations (n = 42).

FIGURE 3 Geographic representation of *Q*-values for each individual (black dot) from K = 2 results from a) STRUCTURE (run with ALPHA = 0.50 and FREQSCORR = 1) and b) TESS. Mexico Beach, Florida is depicted by a yellow star. Note that although the coloring encompasses the entire marine landscape, some caution should be taken when interpreting population ranges in areas from which no samples were analyzed (e.g., within most bays, sounds, estuaries, and in waters > 200 m).

FIGURE 4 Median-joining network showing the relationships among mtDNA haplotypes. Each haplotype is represented by a circle and sized proportionally based on the number of individuals sharing that haplotype. The haplotype circles are proportionally color-coded based on their frequency found in the green and/or blue populations. Black diamonds represent unsampled or ancestral haplotypes. One mutation separates each haplotype unless otherwise denoted. The five

haplotypes that correspond to the offshore ecotype are encircled in a red dashed line. All other haplotypes are of the coastal ecotype.

FIGURE 5 Map of all bottlenose dolphin samples assigned to either the green or blue population based on STRUCTURE results (ALPHA = 0.50 and FREQSCORR = 1) overlaid with cumulative surface oil coverage in cubic meters (m^3) resulting from the *Deepwater Horizon* oil spill. Cumulative surface oil is a measure of the combined floating surface oil and oil emulsion detected from April 24 – August 3, 2010. The darker the square the higher the cumulative amount for the given region. The 20, 40, 60, 80, 100, and 200 m isobaths are shown.



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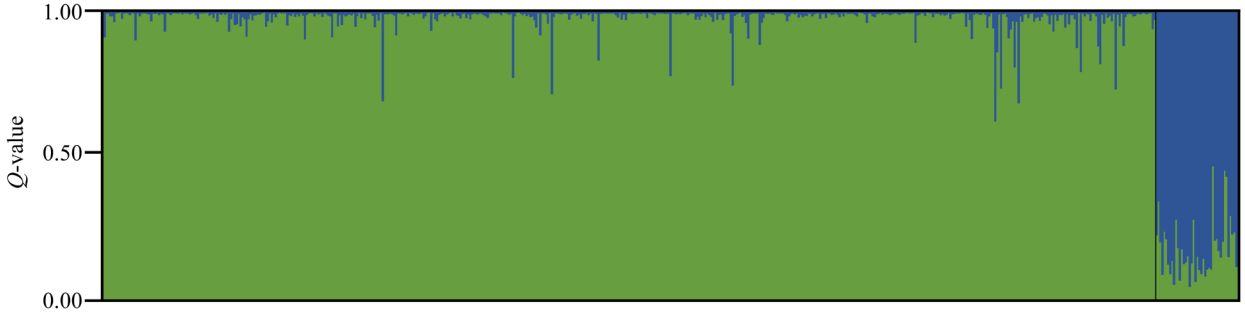

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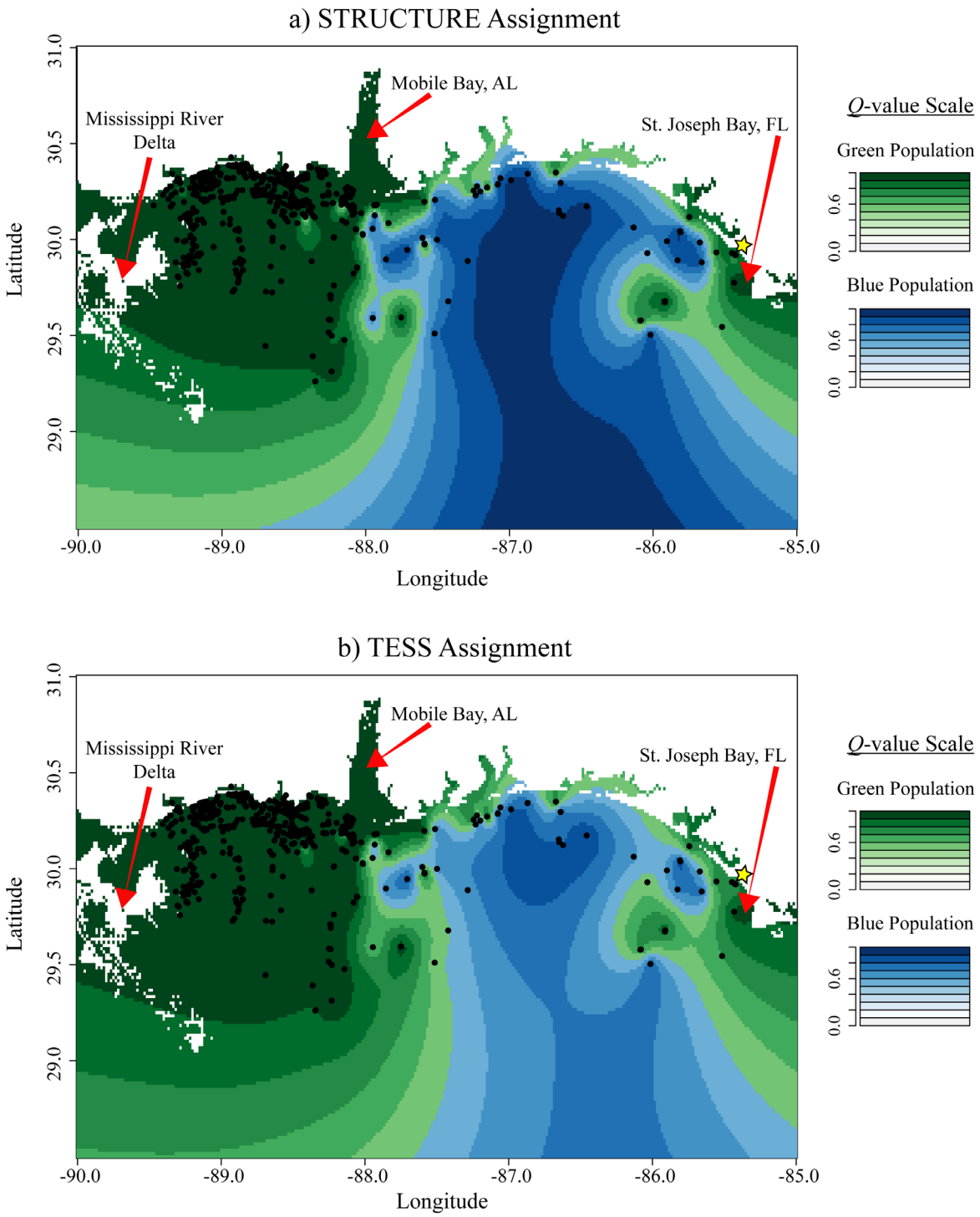

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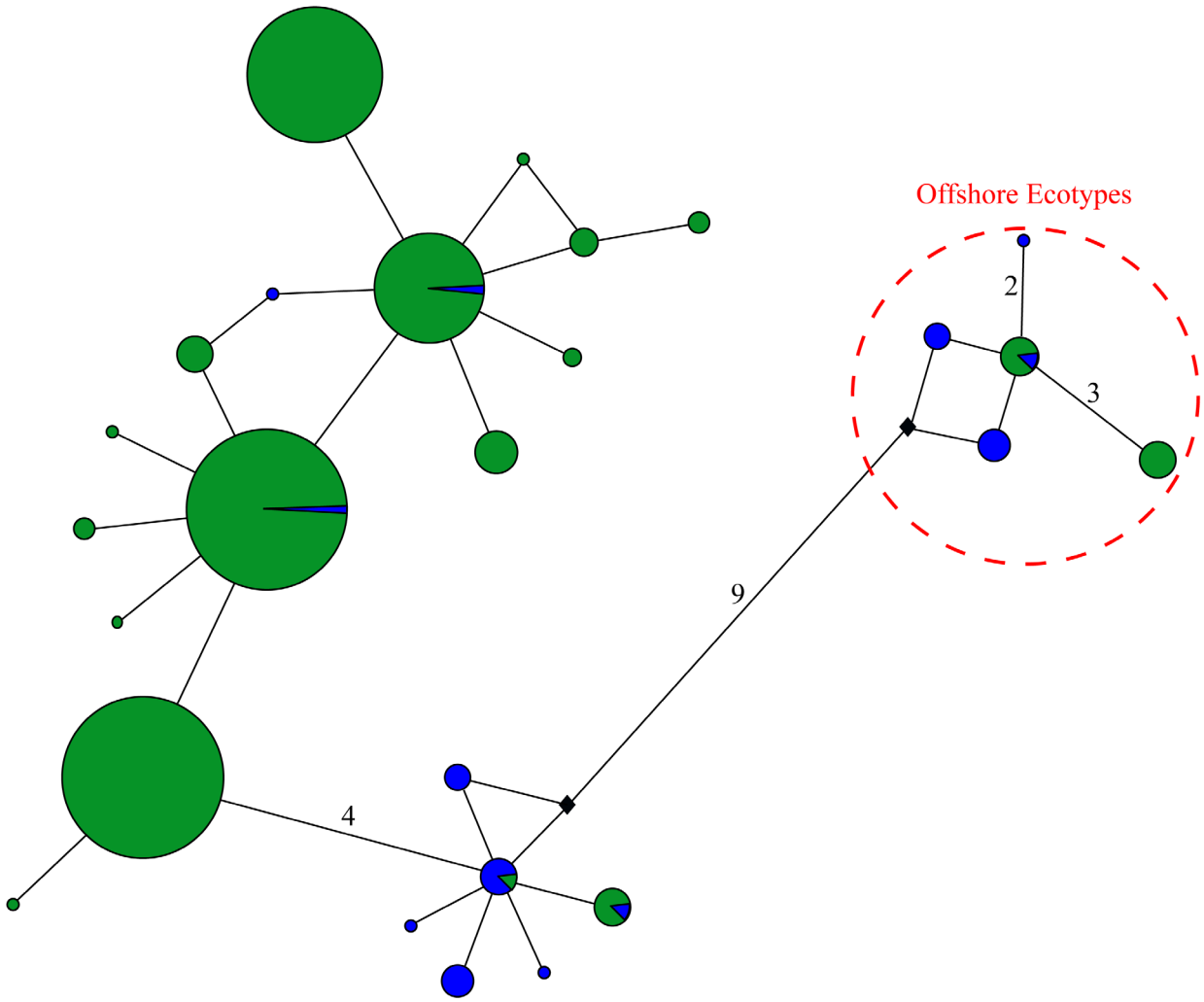

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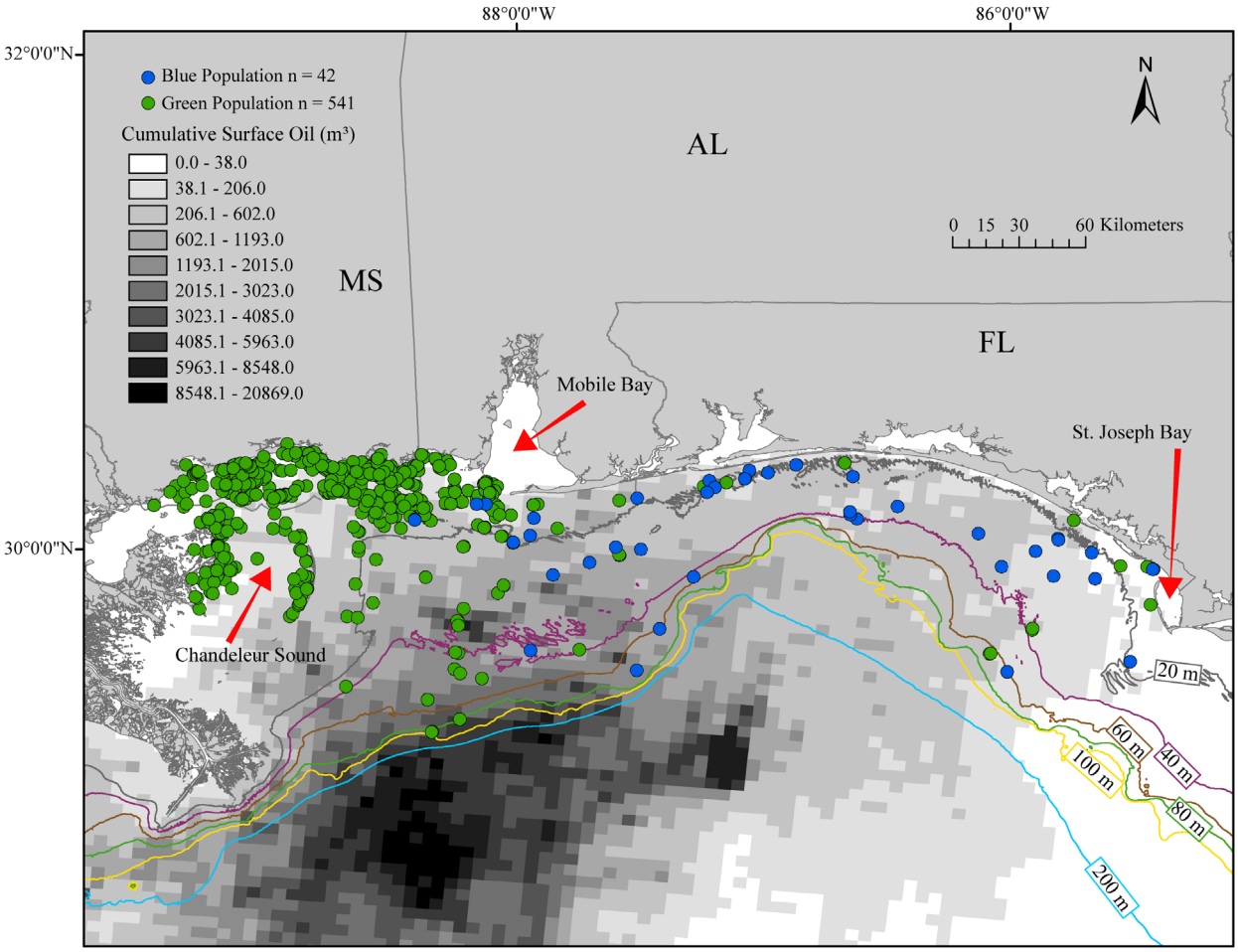

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