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Abstract

Harbor porpoise in the North Pacific are found in coastal waters from southern California to Japan, but population structure is poorly known outside of a few local areas. We used multiplexed amplicon sequencing of 292 loci and genotyped clusters of SNPs as microhaplotypes (N=271 samples) in addition to mtDNA sequence data (N=413 samples), to examine the genetic structure from samples collected along the Pacific coast and inland waterways from California to southern British Columbia. We confirmed an overall pattern of strong isolation-by-distance, suggesting that individual dispersal is restricted. We also found evidence of regions where genetic differences are larger than expected based on geographic distance alone, implying current or historical barriers to gene flow. In particular, the southernmost population in California is genetically distinct (F_{ST} = 0.02 (microhaplotypes); 0.31 (mtDNA)), with both reduced genetic variability and high frequency of an otherwise rare mtDNA haplotype. At the northern end of our study range, we found significant genetic differentiation of samples from the Strait of Georgia, previously identified as a potential biogeographic boundary or secondary contact zone between harbor porpoise populations. Association of microhaplotypes with remotely-sensed environmental variables indicated potential local adaptation, especially at the southern end of the species' range. These results inform conservation and management for this nearshore species, illustrate the value of genomic methods for detecting patterns of genetic structure within a continuously distributed marine species, and highlight the power of microhaplotype genotyping for detecting genetic structure in harbor porpoises despite reliance on poor-quality samples.

Key Words: sPCA, dbRDA, seascape genetics, mtDNA, SNP, microhaplotype, GT-seq, cetacean

Introduction

Harbor porpoises (*Phocoena phocoena*) are small cetaceans found in temperate and sub-Arctic coastal waters, typically in less than 200m depth (Fontaine, 2016; Read, 1999) (but see Nielsen et al., 2018). Their range is considered continuous along the continental coasts, but there is high variability in density and apparent gaps in suitable habitat (Evenson, Anderson, Murphie, Cyra, & Calambokidis, 2016; Forney, Moore, Barlow, Carretta, & Benson, in press). They are susceptible to entanglement in gillnets and disturbance from construction activities for wind farms (Carstensen, Henriksen, & Teilmann, 2006; Reeves, McClellan, & Werner, 2013). In addition, the linear coastal distributions with gaps raises the question of how harbor porpoise will respond to climate change. Areas with sufficient data indicate fine-scaled population structure is common (Chivers, Dizon, Gearin, & Robertson, 2002; Crossman, Barrett-Lennard, & Taylor, 2014; Fontaine, 2016; Lah et al., 2016; Rosel, France, Wang, & Kocher, 1999; Tiedemann, Harder, Gmeiner, & Haase, 1996; Walton, 1997; Wang & Berggren, 1997). Along the U.S. west coast, early genetic studies based on mitochondrial DNA (mtDNA) provided data to delineate management stocks (Chivers et al., 2002), but data gaps made boundary placement difficult. Because harbor porpoises avoid vessels, which makes dart-biopsy an unviable method to obtain sufficient samples, most samples are from either beach-stranded animals or entangled animals from areas with gillnet fisheries. As a result, sampling gaps existed in areas without gillnet fisheries and samples were often obtained days after death and in moderate to advanced stages of decomposition, and hence had degraded DNA.

Although gillnetting has declined along the U.S. west coast, mitigation of other potential threats, such as development of wind farms, requires an understanding of population structure, often in areas poorly sampled in previous studies. The U.S. Marine Mammal Protection Act (MMPA) manages at the scale of demographically independent populations (DIPs) where allele (or mtDNA haplotype) frequencies will differ but evolutionary differences are not expected. Because our area of interest includes the southernmost part of harbor porpoise distribution in the Pacific where ocean temperatures

93 are already rising, an understanding of evolutionary barriers to dispersal is also of interest
94 to evaluate potential impacts of such temperature shifts.
95
96 In our study area along the U.S. West Coast and inland waters of Washington, samples
97 have slowly accrued to fill most gaps over a period of 30 years, but most samples are of
98 degraded quality and sample size remains low in some areas. An additional complication
99 requiring consideration for marker choice is that there is evidence of intergeneric
100 hybridization with Dall's porpoise (*Phocoenoides dalli*) in the eastern North Pacific
101 (Baird, Willis, Guenther, Wilson, & White, 1998; Crossman et al., 2014; Willis, Crespi,
102 Dill, Baird, & Hanson, 2004), which could affect population analyses, especially if
103 hybridization has been regionally restricted and/or resulted in introgressive gene flow
104 between species.
105
106 Genomic methods such as genome re-sequencing (e.g., Foote et al., 2019) and reduced
107 representation sequencing (e.g., Andrews, Good, Miller, Luikart, & Hohenlohe, 2016;
108 Maisano Delser et al., 2016) can produce thousands to tens of thousands of genetic
109 markers, providing unprecedented power to detect population differences (e.g., Candy et
110 al., 2015; Emerson et al., 2010; Leslie & Morin, 2016) and identify candidate loci under
111 selection (Ahrens et al., 2018). However, for studies restricted to the use of poor quality
112 archived samples, the optimal strategy involves targeting a reduced number of genetic
113 markers that can efficiently and reproducibly be obtained from a large number of
114 samples, such as "genotyping in thousands by sequencing" (GT-seq; Campbell, Harmon,
115 & Narum, 2015). Typically targeting a few hundred loci, GT-seq relies on multiplexed
116 short amplicon sequencing that requires only ~20ng of DNA, and locus variability can be
117 increased by targeting highly variable regions with multiple single nucleotide
118 polymorphisms (SNPs) genotyped as microhaplotypes (Baetscher, Clemento, Ng,
119 Anderson, & Garza, 2017; McKinney, Seeb, & Seeb, 2017).
120
121 To increase power to detect population structure from larger numbers of SNPs, while
122 maximizing our ability to use an existing collection of poor-quality samples collected
123 over three decades, we employed GT-seq multiplex sequencing combined with

124 microhaplotype analysis, and traditional mtDNA control region sequencing to incorporate
125 new and previously published data. We describe population structure at both evolutionary
126 and demographic scales through a continuous range in the eastern North Pacific by
127 applying a combination of Bayesian assignment to detect more substantial divergence of
128 evolutionarily significant units (ESUs; Moritz, 1994), and traditional divergence metrics
129 and spatially explicit methods to detect demographically independent populations (DIPs),
130 the basis of marine mammal population stocks under the U.S. Marine Mammal Protection
131 Act (Martien et al., 2019; Waples & Gaggiotti, 2006). Evolutionarily significant units are
132 expected to have very low gene flow (on the order of a few successful dispersers per
133 generation) and significant differences in both nuclear and mitochondrial markers. In
134 contrast, a demographic independence means that the population dynamics of the affected
135 group is more a consequence of births and deaths within the group (internal dynamics)
136 than immigration or emigration (external dynamics) (Martien et al., 2019). DIPs could
137 have gene flow on the order of 1-2% per year. Besides the large difference in gene flow,
138 DIPs could be based solely on mtDNA because birth and death rates depend on females,
139 though use of larger numbers of nuclear markers may provide higher statistical power to
140 detect population differences.
141
142 We also use seascape genotype-environment association to investigate patterns of
143 localized adaptation. We anticipate that these genomic scale data will not only provide
144 increased resolution to detect spatial structure among populations, but also allow us to
145 link genotypes to environmental variables that vary both spatially and temporally. This
146 emerging field of seascape genomics (Riginos, Crandall, Liggins, Bongaerts, & Treml,
147 2016) is still in its early stages relative to landscape genomics, due to both the difficulty
148 of broad scale sampling of many marine species and the limited availability of relevant
149 environmental predictors. However, the increasing accessibility of remotely-sensed
150 oceanographic variables has improved our ability to identify spatial, temporal, and
151 ecological factors that promote population structure and local adaptation in complex and
152 dynamic seascape environments (reviewed by Riginos et al., 2016; Selkoe et al., 2016).
153
154 **Materials and Methods**

155

156 **Samples**

157 Harbor porpoise (N=441), Dall's porpoise (N=9) and putative Harbor/Dall's porpoise
158 hybrids (N=13) skin samples were collected from beach-cast carcasses, carcasses
159 recovered as fisheries bycatch, or from animals live-captured for tagging. Tissue samples
160 were preserved in salt-saturated 20% DMSO or 100% ethanol and subsequently stored at
161 -20°C in the U.S. National Marine Fisheries Service (NMFS) Marine Mammal and Sea
162 Turtle Research (MMASTR) Collection at the Southwest Fisheries Science Center
163 (SWFSC). Sample information is in supplemental Table S1 and locations are shown in
164 Figure 1. DNA was extracted from tissue samples using a variety of common extraction
165 methods, including silica-based filter membranes (Qiaxtractor® DX reagents, Qiagen,
166 Valencia, CA, USA), standard phenol/chloroform extraction (modified from Sambrook,
167 Fritsch, & Maniatis, 1989), lithium chloride (Gemmell & Akiyama, 1996) and sodium
168 chloride protein precipitation (Miller, Dykes, & Polesky, 1988), and quantified using the
169 Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Paisley, UK) with a Victor X3
170 fluorospectrometer (Perkin Elmer, Waltham, MA, USA).

171

172 **mtDNA control region sequencing**

173 New 395bp mitochondrial DNA control region sequences (N=176) were generated
174 according to previously published methods for harbor porpoise studies (Chivers et al.,
175 2002; Chivers et al., 2007; Crossman et al., 2014). Electropherograms of sequences from
176 previous studies (N=224 from Chivers et al. 2007; N=91 from Crossman et al. 2014)
177 were evaluated and compared to newly generated sequences, and regions with
178 ambiguities or poor quality were either re-evaluated by a single person to ensure
179 consistent interpretation (N=224), or the DNA was sequenced again from new PCR
180 reactions using current Sanger sequencing chemistry (N=91). Haplotype IDs were
181 assigned using the LabelHaplotypes function in the R package *strataG* (v2.4.905; Archer,
182 Adams, & Schneiders, 2017) and associated with previously published sequences and
183 haplotypes (supplemental Table S2).

184

185 **SNP discovery**

DNA from 12 North Pacific harbor porpoises was used for indexed genomic library preparation using the Accel-NGS 2S PCR-free genomic library preparation kit (Swift Biosciences, Ann Arbor, MI, USA). Either 100 ng or 500 ng of genomic DNA, determined by Qubit (Thermo Fisher Scientific, Waltham, MA, USA) quantification, was used for the library prep to obtain sufficient library product to pool 15 nmol/l of each sample prior to sequencing. For 12 North Atlantic samples, genomic DNA was used for indexed genomic library preparation at BGI (Hong Kong) following their proprietary protocol and pooled for next generation sequencing (NGS). The North Pacific pooled library was first sequenced in two 150 bp, paired-end Illumina, Inc. (La Jolla, CA, USA) MiSeq lanes, then both pooled libraries were sequenced in two Illumina HiSeq-4000 lanes each.

NGS read data were trimmed and filtered using Trimmomatic (v. 0.36; Bolger, Lohse, & Usadel, 2014) to remove Illumina adapters, reads <50bp and low-quality bases (Q<20) at the beginning and end of reads. The sliding window approach was implemented to change internal bases with Q<15 (sliding window of 4bp) to N's. Because there was some bias in the nucleotide frequencies, the first 4 bp of all sequences were also removed.

Genome alignment and SNP discovery were conducted as previously described (Morin et al., 2018). The repeat-masked killer whale genome (accession GCA_000331955.2; Foote et al., 2015) was used as the reference for genome assembly. Briefly, the paired-end MiSeq reads from the 12 North Pacific samples were assembled *de novo* using CLC Genomics Workbench (v.4.1; CLCbio) to obtain a complete reference mitochondrial genome as previously described (Hancock-Hanser et al., 2013). The NGS reads from 12 samples from each ocean basin subspecies were aligned separately to the harbor porpoise mitochondrial genome using BWA mem (v. 0.7.5a; Li & Durbin, 2009) and non-aligned reads were extracted using samtools (v. 1.2; Li et al., 2009). The extracted (non-mitochondrial) reads from each sample were separately aligned to the killer whale reference genome, combined into one alignment, and the consensus harbor porpoise genome sequence generated. The nuclear DNA reads for each sample were aligned to the

new harbor porpoise consensus genome sequence as above, followed by SNP discovery separately from each subspecies using GATK (v. 2.5-2; DePristo et al., 2011; McKenna et al., 2010). Potential SNPs were filtered to remove SNPs with mapping quality <30, excessive coverage (>2x mean depth of coverage), and estimated minor allele frequency <0.05. To avoid linked loci, SNPs were selected from contigs that were at least 100 kb in length, and SNPs were at least 100 kb apart on the contigs. SNP loci from the two subspecies were compared to identify loci that were polymorphic in both ocean basins to avoid ascertainment bias in application to either subspecies. Finally, candidate SNPs were compared to GenBank using BLAST+ (Camacho et al., 2009) and filtered to remove loci that were potentially in repeat regions, gene families, or a close match to non-mammalian species. The resulting set of filtered SNPs are subsequently referred to as "targeted SNPs", as these were the initial targets for our SNP genotyping effort. Additional SNPs detected in short GT-seq sequences were combined with the targeted SNPs to form microhaplotype genotypes.

Multiplex primer design

Primers were designed from a batch of 500 loci selected randomly from the filtered SNPs using the program FastPCR (Kalendar, Lee, & Schulman, 2011). Parameters for primer selection (from 300 bp sequences with the target SNP at position 151) were: primer length = 15-32, tm = 57 – 62, 3' Tm = 25-50, dimer stringency = 5, synchronized Tm for primer pair = 5, Forward primers between position 40 and 150, Reverse primers between position 152 and the 3' end, and addition of 5' GT-seq tails for indexing and library preparation: F-tail = CGACAGGTTCAGAGTTCTACAGTCCGACGATC, R-tail = GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT. After primer design, all loci with both forward and reverse primers were compared using the "Primers list analysis" function in FastPCR to detect cross-locus primer dimer interactions with Tm > 20°C ("strong" primer dimers). Loci were filtered out of the primer list if either one or both primers had predicted interactions with Tm > 40°C with >2 other primers, or if the predicted primer interactions were >46°C. Primers for 385 loci were synthesized at 100 μM concentration in 96-well plates by Integrated DNA Technologies (Coralville, Iowa, USA).

Multiplex PCR optimization

GT-seq primers were pooled and used for multiplex amplification of one sample initially to optimize the locus set prior to genotyping. Optimization consisted of multiple rounds of GT-seq library preparation as described by Campbell et al. (2015), and sequencing a small portion of the library (e.g., 1-10 million reads) to determine the relative abundance of reads per locus, and presence of primer artifacts as determined by the published analysis scripts (https://github.com/GT-seq/GT-seq-Pipeline). Loci were removed at each iteration to eliminate loci represented by disproportionately high read depths, evidence of primer artifacts, or low ratios of the probe to primer target sequences. There are no published guidelines for cut-off values, so we removed loci that appeared to be outliers for any of these values, and based on expert advice from experienced users of the GT-seq method (see acknowledgements). Primer sequences for the final set of 292 loci used for genotyping are in supplemental Table S3.

SNP genotyping

Amplicon libraries were prepared following the GT-seq protocol, including the optional Exo-SAP pre-treatment of the samples (Campbell et al., 2015), and pooled libraries were sequenced on an Illumina NextSeq500 sequencer, 1x150 bp reads. Custom scripts for processing GT-seq data (Campbell et al., 2015) were used to demultiplex the sample files and conduct preliminary genotyping. Genotypes were quality checked for duplicate samples, percent missing genotypes per locus and sample, and percent homozygosity using the *strataG* package in R. Replicate samples were used to estimate genotyping error rates, then fastq files from replicates samples were combined to a single file per sample.

Fastq files were checked for standard quality metrics (e.g., per base quality scores, nucleotide composition, sequence duplication level, overrepresented sequences) with FASTQC v0.11.3 (Babraham Bioinformatics), then trimmed using FASTP (Chen, Zhou, Chen, & Gu, 2018) to remove adapter sequences and poly-A and poly-G 3' tails that were added during sequencing of amplicons shorter than 150 bp, and to exclude reads shorter than 30bp after trimming. Reads were mapped to the reference locus sequences using the

BWA MEM algorithm (v. 0.7.15; Li & Durbin, 2009), and SNPs detected across all samples using FREEBAYES v1.1.0-54-g49413aa (Garrison & Marth, 2012) after removing sample files smaller than 1 MB (containing <0.1 M filtered reads). FREEBAYES was run with minimal filtering (supplemental materials), followed by additional filtering with vcfTools v0.1.12b (Danecek et al., 2011) to extract the targeted SNP for each locus (for targeted SNP analysis; see below), and to remove sites with low coverage (minimum depth = 10), indels, and loci with less than 30% completed genotypes.

For targeted SNPs (position 151 in all reference sequences), custom scripts in R (supplemental materials) were used to extract the genotype data from the vcf file, generate allelic count plots to visualize the genotype distributions of reads for each allele, and re-call genotypes based on minimum depth and allelic ratios. The minimum depth of 10 reads total and default minor allele read proportion for heterozygotes of >0.3 were adjusted as needed until genotypes clearly fell into separate clusters in the allelic plots. Loci with poor resolution of plotted genotypes were removed from the data set.

Microhaplotypes (containing the targeted SNPs and/or newly discovered SNPs) were generated for all loci using the R package *MicrohaPlot* (Baetscher et al., 2017). The *MicrohaPlot* algorithm inserts N's for missing sequence data at SNPs within haplotypes, so we used a custom R-scripts (supplemental materials) to identify SNPs with >10% N's. The identified SNPs were removed from the original vcf file using vcfTools, and *MicrohaPlot* was used to generate new microhaplotypes with the remaining variable SNP positions. The unfiltered haplotypes were exported for subsequent filtering with custom scripts to view and call genotypes similar to the methods described above for targeted SNPs (supplemental materials). The few remaining microhaplotypes with N's in them were excluded from genotypes prior to analysis.

A final combined data set for microhaplotypes and targeted SNPs was created by combining the multi-SNP loci with the single-SNP (targeted) loci. Since the targeted SNP loci had been genotyped using two different methods, we selected the genotype data for

each locus from the method that provided the higher quality or quantity of genotypes for the targeted SNP. Some microhaplotype loci that were monomorphic in harbor porpoises, or which had similar genotype quality for the targeted SNP but contained other SNPs present in Dall's porpoise samples, were retained (instead of the targeted locus data) to allow genetic identification of intergeneric hybrids. All loci that were found only in the microhaplotype or targeted SNP data set were then added to the filtered loci to generate a final data set.

Quality analysis

Quality analysis and sample and locus filtering were conducted using custom R scripts. Samples missing >80% of the genotypes, and loci missing more than 45% of the genotypes were also removed. Genetic duplicates (>80% identity) were identified and one from each pair of samples identified as duplicates was removed. Analyses of deviations from Hardy-Weinberg equilibrium (HWE) expectations were conducted across all samples using the R package *adegenet*, and loci with a difference between observed and expected heterozygosity >0.2 were removed as extreme outliers (>10x the average difference of 0.02), most likely due to non-mendelian loci (e.g., null alleles, duplicated loci, or high error rates). Remaining loci were tested for significant linkage disequilibrium (LD) and deviations from HWE within three discrete, geographically-defined strata represented by greater than 20 samples (inland waterways (N=88), Neah Bay (N=21), and Northern California/Southern Oregon (N=35)), after correction for multiple tests using a sequential correction (Holm, 1979). We used the R package *Demerelate* (v. 0.9.3; Kraemer & Gerlach, 2017) using relatedness estimators "Wang" and "Mxy" to test for inadvertent sampling of close relatives.

Porpoise distribution data

To examine the genetic results in the context of harbor porpoise distribution and relative density along the U.S. West Coast, independent aerial survey data collected during 1991-2017 off California (e.g., Forney, Hanan, & Barlow, 1991), and during 1989-2003 off Oregon and Washington (e.g., Calambokidis, Laake, & Osmek, 1997) were processed to derive the number of porpoise seen per kilometer surveyed as an index of relative

density. Although the transect design differed between these two data sets, the survey protocols, observer team and configuration, and aircraft type were the same. Survey data were truncated spatially to include only the primary porpoise habitat extending from shore out to 90-100 m water depth. Relative densities were calculated for each transect line, assigned to latitude of the transect mid-point, and then smoothed south-to-north using a Loess smoother.

Habitat data

A variety of modeled and measured oceanographic variables (Table 1) were extracted to examine potential environmental correlates of genetic patterns. These predictors included 1) sea surface temperature, sea surface height, mixed layer depth, and the standard deviation of these three variables derived from the Regional Ocean Modeling System (ROMS) outputs (Moore et al., 2011), 2) coastal upwelling indices (Jacox, Edwards, Hazen, & Bograd, 2018), and 3) multispectral ultra-high resolution sea surface temperature and its standard deviation (Chin, Vazquez-Cuervo, & Armstrong, 2017).

Analytical methods

Several methods were used to estimate the number of populations and population assignment based on the genetic data. Population structure and individual assignment was examined using STRUCTURE (v. 2.3), which implements a Bayesian clustering method to identify significant genetic clusters based on Hardy-Weinberg equilibrium allele frequency expectations (Hubisz, Falush, Stephens, & Pritchard, 2009; Pritchard, Stephens, & Donnelly, 2000). We ran ten replicates for each value of k (where k is the number of putative populations), using correlated allele frequencies and an admixture model, with location prior using geographically defined units (supplemental Table S1). Each analysis consisted of 50,000 burn-in steps followed by 100,000 MCMC steps, and 10 replicates combined using 100 iterations in CLUMPP (v. 1.1.2; Jakobsson & Rosenberg, 2007). We also used CLUMPAK (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015) to assess convergence of the MCMCs and evaluate consistency of replicates across values of K. The ΔK method (Evanno, Regnaut, & Goudet, 2005) was used to evaluate most likely number of inferred clusters.

372
373 STRUCTURE is known to have low power to detect populations (i.e., DIPs) when there
374 is even a very low migration rate (m≥0.005/generation), where demographic
375 independence and biologically meaningful differentiation still exist (e.g., Cullingham et
376 al., 2020; Kalinowski, 2011). To better differentiate DIPs, we examined spatially explicit
377 principal components with geographic information using sPCA (Jombart, Devillard,
378 Dufour, & Pontier, 2008). Spatial distances were based on type 1 (Delaunay
379 triangulation) connection network. We tested for significant evidence of structure in the
380 sPCA using the Eigenvalue test "spca_randtest" (Montano & Jombart, 2017) with 9999
381 permutations in the R package *adegenet* (v. 2.1.1; Jombart, 2008). Geographical subsets
382 of the data were analyzed hierarchically to evaluate structure at decreasing spatial scales.
383
384 Given the nearly continuous distribution of harbor porpoises along a coastline, we tested
385 for genetic isolation by distance using Mantel tests for correlation of both individual and
386 population genetic distances with geographic distances using the *adegenet* R package.
387 We used Euclidean distance for individuals (Euclidean distance among vectors of allele
388 frequencies) and pairwise F_{ST} distances between population strata, and straight-line
389 geographic distances between individual samples or average latitude/longitude position
390 of samples clustered into *a priori* geographic populations. We also used Monmonier's
391 algorithm (Monmonier, 1973) as implemented in *adegenet*. Putative boundaries between
392 populations were inferred based on the default threshold value (third quartile of all
393 distances between neighbors).
394
395 For both nuclear and mtDNA data, we tested for *a priori* population divergence using
396 pairwise estimation of F_{ST} with MCMC resampling implemented in the *strataG* R
397 package. Multiple stratification schemes were tested based on *a priori* information gained
398 from the Bayesian population genetic and sPCA analyses described above, previously
399 defined management stocks (Carretta et al., 2019), and gaps in harbor porpoise
400 distribution (Forney et al., 1991). For mtDNA, we generated a median joining network
401 (MJN) using the program POPART (Leigh & Bryant, 2015).
402

403 We used distance-based redundancy analysis (dbRDA) to investigate genotype-
404 environment associations and identify microhaplotypes potentially under selection
405 (Forester, Lasky, Wagner, & Urban, 2018). dbRDA identifies how groups of SNPs or
406 microhaplotypes covary in response to the multivariate environment. It is well-suited to
407 isolation-by-distance demographic scenarios, maintaining both high true positive and low
408 false positive rates (Forester et al., 2018). Environmental variables (Table 1) were
409 extracted from longitudinal oceanographic data matched to collection date, latitude and
410 longitude of samples. We used Bray-Curtis dissimilarity (Bray & Curtis, 1957) to
411 calculate the microhaplotype distance matrix. This approach quantifies the dissimilarity
412 among individuals based on their multilocus genotypes, and is equivalent to proportion of
413 shared alleles (Shirk, Landguth, & Cushman, 2017). We performed dbRDA separately
414 for porpoises from the outer coastal and inland waterways regions, because data for all
415 environmental predictors were not available for all locations (Table 1).
416
417 For dbRDA, we first removed microhaplotype loci with heterozygosity less than 0.05,
418 then removed individuals with missing data for the retained environmental predictors. We
419 produced three data sets for each region (i.e., outer coastal and inland waterways),
420 representing three thresholds of missing genotype data across individuals: 25%, 20%, and
421 15%. We imputed missing values for each data set using *snmf* in the LEA package v.
422 3.1.2 (Frichot & Francois, 2015), testing values of K from 1-5, and alpha (regularization
423 parameter) values of 10, 100, and 1000. All runs used 25 repetitions, 200 iterations, and a
424 5% cross entropy withholding. We then performed a dbRDA for each imputed data set,
425 retaining three constrained axes for outlier analysis. We identified candidate
426 microhaplotypes under selection using robust (e.g., not sensitive to outliers) Mahalanobis
427 distance (Capblancq, Luu, Blum, & Bazin, 2018), which identifies outlier
428 microhaplotypes based on their constrained ordination loadings in multidimensional
429 space. We accounted for confounding factors in the dbRDA, such as population structure
430 and isolation-by-distance, using the genomic inflation factor (Francois, Martins, Caye, &
431 Schoville, 2016), and applied a false discovery rate cutoff of 0.1 (Storey & Tibshirani,
432 2003) to identify outlier microhaplotypes showing relationships with environmental
433 variation. Finally, we compared detections across missing data thresholds.

RESULTS

We used a total of 431 mtDNA control region sequences (395bp) from previously published (N=363, including re-sequenced samples) and newly generated (N=68) sequences, and SNP data for 296 porpoises ranging from the southern extent of the harbor porpoise range in Southern California, USA, to British Columbia, Canada (Supplemental Table S1, Figure S1). Resequencing of samples with previously published haplotypes resulted in ten haplotype changes from the Chivers et al. (2002; 2007) studies, and 53 from Crossman et al. (2014), mostly due to resolution of ambiguous positions in the previous electropherograms that resulted in synonymization of multiple haplotypes from each of those studies. We identified 52 harbor porpoise haplotypes, 22 of which were found in only a single individual, and six Dall's porpoise haplotypes (supplemental Tables S4, S5). Our GT-seq locus panel consisted of 340 loci, of which 290 were genotyped in at least 55% of the samples and were polymorphic in harbor porpoises. Two additional loci were polymorphic only in Dall's porpoises and were used to identify hybrids between the 2 species. Of the 290 loci, 151 (52%) contained a single SNP, while the remaining 139 (48%) contained ≥2 SNPs, genotyped as microhaplotypes. None of the loci deviated significantly from HWE, and significant LD was only detected in one locus pair in one of the three tested geographic strata; no loci were removed based on these tests of HWE or LD.

DNA quantity and quality varied substantially among samples, resulting in variable number of completed genotypes, and an inverse correlation of error rate with the number of completed genotypes. Arbitrarily changing the cut-off value for percent completed genotypes can result in slight changes (see error rates below) to overall data quality, but at the cost of reduction in sample sizes in individual strata, reducing statistical power to detect structure. To maximize the sample sizes across strata, we used 20% (≥58 of 292) genotyped loci as the minimum cut-off, resulting in 296 genotyped samples (after removal of unintentional duplicates (see below): 280 harbor porpoise, 11 Dahl's porpoise,

5 hybrids). Of the harbor porpoise samples, 72% were genotyped at >90% of the loci, and 83% were genotyped at >80% of the loci (supplemental Table S1).

Average per-allele error rates for SNPs were calculated from 32 samples genotyped in duplicate from separate GT-seq amplicon libraries, based on single SNPs in 270 loci that were genotyped in >50% of samples. Intentionally replicated samples for which there was sufficient data in both replicates (N=32) had matching genotypes at an average of 96% of the loci (range 87-100%). For samples genotyped at >80% of the loci, the estimated error rate (based on 23 replicated sample pairs) was 0.010/allele. For samples with lower genotype completion rates (46 in the harbor porpoise data set), the mean error rate estimate increased to 0.045/allele (based on N=9 replicate pairs). Nine sample pairs (excluding Dall's porpoise samples, which were too homozygous in this data set) were identified as unintentional duplicates based on at least 85% identical genotypes (range 96-100%), and one from each pair was removed from further analysis. All of the genetic duplicates were inadvertent duplicate samples from the same individual, usually due to sample being stored in different collections with different identification codes. We detected three potential first order relatives (full siblings or parent-offspring pairs) based on the "wang" estimator, and six (including two of the three from the "wang" estimator) based on the "Mxy" estimator (supplemental Table S6) (see Kraemer & Gerlach, 2017, for details). All samples were retained for some of the subsequent analyses, but one individual from each putative pair was removed to control for the effects of sampling closely related individuals in STRUCTRURE, sPCA, and F_{ST} analyses.

Putative hybrids between parapatric Dall's and harbor porpoise species have been previously identified based on phenotype and genetic profiles (Crossman et al., 2014). We genotyped nine known Dall's porpoises and had phenotypic or previous genotypic indication (based on microsatellites; Crossman et al., 2014) of 11 putative hybrids in our final data set. Assignment analysis of all samples using STRUCTURE with the number of clusters set to k = 2 correctly assigned all nine Dall's porpoises to one group, and additionally assigned two of the putative hybrids to the same group with 100% probability. Five of the putative hybrids were assigned to the harbor porpoise group with

≥99.9% probability, and four samples were identified as F1 hybrids with assignment probabilities to each species group between 45% and 55%. One additional sample previously identified as a harbor porpoise was also identified as an F1 hybrid (49%/51% assignment to the 2 species groups). The admixture plot from ten combined structure analyses is presented in supplemental Figure S2a. All F1 intergeneric hybrids were from samples collected in the San Juan Islands and Oregon-Washington coast geographic strata, between latitudes 47º and 49º N. All remaining putative harbor porpoise samples were assigned to the second group with >99.6% probability. As the targeted SNP loci were ascertained only from harbor porpoise samples, Dall's porpoise samples had unsurprisingly low diversity (Table 2), but the use of additional SNPs in microhaplotypes provided variable loci useful for species assignment and hybrid identification. Samples identified as hybrids were removed from subsequent harbor porpoise analyses.

STRUCTURE is useful for identifying population differentiation at the ESU level, where divergence is sufficient to allow high probability of assignment of samples to populations or clusters (Waples & Gaggiotti, 2006). We started with STRUCTURE in a hierarchical analysis of the SNP data to identify evolutionarily divergent populations within our harbor porpoise sample distribution. Structure analysis with all samples assigned to geographic strata *a priori* (supplemental Table S1) did not provide strong evidence of multiple divergent groups along the U.S. west coast, with the number of groups most likely ≤2 (based on ΔK and CLUMPAK similarity score >0.993 for 10/10 replicates for K1-2; supplemental Figure S2b). However, assignment probabilities among three groups differed substantially across geographic region, especially in the three southernmost geographic strata (Figure 2). Subsets of samples representing only high-quality samples (<80% complete) and smaller geographic regions did not result in any additional evidence of population structure (supplemental Figures S2c-e).

Spatial principle component analysis (sPCA; Jombart et al., 2008) has been shown to be a useful tool for detecting patterns of genetic variability in harbor porpoise (Fontaine et al., 2017; Lah et al., 2016), and was used to explicitly combine geographic and nuclear genetic data and to investigate spatial patterns of genetic variation without assumptions of

Hardy-Weinberg expectations or linkage disequilibrium (Montano & Jombart, 2017). Plots of spatial principle components across the study range indicated strong evidence of structure (spca-randtest $p < 0.001$) and show clear separation between samples representing the two southern-most strata, Morro Bay and Monterey Bay, along the first axis (PC1, Figure 3A, 3B), with less separation but a north-south gradient along spatial PC axes two and three (Figure 3A; supplemental Figure S3A). Additional geographic population structure is revealed by analyzing geographic subsets of the data. Hierarchical analysis of sub-regions reveals population structure within the inland waterways ($p = 0.004$; Figure 3G), and within the Washington inland waters managment stock (excluding the Strait of Georgia strata samples) ($p = 0.019$; supplemental Figure S3H). sPCA of samples from coastal waters (Figure 3B), excluding inland waterways in Washington and British Columbia, shows strong evidence of structure ($p < 0.001$), with the first two spatial PC's highlighting unique clusters corresponding to Morro Bay (PC1), and to a lesser extent the remaining strata along PC2. Hierarchical analysis of subsets of neighboring strata indicated significant structure in analyses of all neighboring strata pairs and within the northern California/southern Oregon management stock ($p = 0.017$; Figure 3D; supplemental Figure S3D). Plots of the first four individual spatial PCs for these stratification sets are in supplemental Figure S3. Replicate analyses based on the smaller number of high-quality samples (>80% complete genotypes) showed similar patterns, but resulted in non-significant p-values in some comparisons (Inland waters, southern OR/OR-WA, and northern California/southern Oregon; Supplemental Figure S3).

A Mantel test supported ($p < 0.001$) isolation by distance (IBD) for nuclear SNP data along the Pacific coast (excluding inland waterways) for individual distances. A scatterplot of genetic and geographic distances (Supplemental Figure S4) shows discontinuities suggestive of differentiated populations rather than continuous clines of genetic differentiation. The Monmonier's algorithm was used to infer locations of genetic boundaries or discontinuities, and identified the major boundary between coastal Washington and western Vancouver Island at the default threshold level (Figure 4A), with lesser boundaries between Morro Bay and Monterey Bay, and Monterey and SF-RR

on the outer coast. Within the inland water ways, the major discontinuities were in the southern Strait of Georgia (Figure 4B). It is not straightforward to interpret the Monmonier plots because the samples are not distributed broadly in two dimensions, but the threshold arrows indicate regions where genetic distances appear to be greater than expected based on geographic distance (see Blair et al., 2012 for discussion of methods of detecting barriers to gene flow).

Genetically similar groups (Figure 3) and IBD discontinuities (Figure 4) coincided closely with several previously defined geographical strata (Figure 5; Chivers et al., 2002; Chivers et al., 2007). Genetic differentiation between adjacent *a priori* strata was tested using pairwise divergence (F_{ST}), for both nuclear data and mtDNA (Table 3). Results are presented based on inclusion of all samples, but pairwise divergence among adjacent strata after removal of one from each pair of potential first order relatives, and after removal of samples with <50% or <80% completed genotypes, did not qualitatively alter results (supplemental Table S7). As expected when larger numbers of loci are used to calculate F_{ST} (Willing, Dreyer, & van Oosterhout, 2012), the F_{ST} point estimates did not change substantially (<0.004), but exclusion of samples reduced the sample sizes in some geographically-defined strata, resulting in loss of statistical power to detect structure among some non-adjacent strata (supplemental Table S7D-F). Along the outer coast the only genetic distances between neighboring strata pairs that were significant ($p < 0.05$) for both mtDNA and nuclear DNA were between Morro Bay at the southern end of the distribution and Monterey Bay ($F_{ST} = 0.020$ (nDNA); 0.310 (mtDNA)). Nuclear locus frequencies were also significantly different between three of the more southern strata pairs (Monterey/SF-RR ($F_{ST} = 0.005$), N. CA/S. OR ($F_{ST} = 0.008$), S. OR/ORWA coast ($F_{ST} = 0.006$), while mtDNA haplotype frequencies were significantly different between only the two northern-most coastal strata pairs (ORWA coast /Spike Rock ($F_{ST} = 0.049$), and Spike Rock/W. Vancouver Is. ($F_{ST} = 0.128$)). Frequency plots of the common mtDNA haplotypes (excluding haplotypes that occurred in fewer than 5 samples) show clear differences among *a priori* geographical strata (Figure 5, supplemental Figure S5), with near fixation of haplotypes in Morro Bay to the south, and private haplotypes in BC to the north. Relative density data showed low-density regions along the Big Sur

588 coastline (35.5-36.5º N), just south of 38º N, and just north of 39º N at the existing
589 management stock boundary. These low relative density areas correspond to existing
590 boundaries between the four southern-most management stocks. The reason for low
591 density for one region, the Big Sur coastline, is likely due to the very narrow shelf, but
592 reasons for density variation in other regions remain unclear.
593
594 Inland waters strata were not differentiated by nuclear marker frequencies except for the
595 Strait of Georgia, which was significantly differentiated from all inland waters and outer
596 U.S. coast strata, but not W. Vancouver Is. or BC (Table 3). The inland waters strata were
597 differentiated from the nearest coastal strata in mtDNA (F_{ST} between 0.023 and 0.247,
598 with most point estimates being significant at $p < 0.05$). Within the inland waters,
599 divergence in mtDNA was significant for Neah Bay, in the outer Strait of Juan de Fuca,
600 versus San Juan Islands ($F_{ST} = 0.066$), and from both neighboring coastal strata (F_{ST} =
601 0.141 (Spike Rock); 0.247 (W. Vancouver Island)). Puget Sound, which has been re-
602 colonized in the last 18 years (Evenson et al., 2016), was not significantly differentiated
603 from either the neighboring San Juan Islands, or Neah Bay in the Strait of Juan de Fuca.
604 The genetic discontinuity identified immediately north of the U.S.-Canadian border at
605 approximately 49.1°N. latitude (Figure 4) warranted an adjustment of the boundary
606 between the Strait of Georgia and San Juan Islands strata from 48.8° to 49.1°, resulting in
607 reassignment of ten and five samples (mtDNA, nDNA analyses, respectively) from the
608 Strait of Georgia to the San Juan Islands strata (Figure 4B). Genetic divergence remained
609 significant following the reassignment of these samples and F_{ST} increased for both nDNA
610 (0.005 to 0.006) and mtDNA (0.095 to 0.153).
611
612 For the genotype-environment association analyses (dbRDA), we analyzed three data sets
613 for each region (i.e., outer coastal and inland waterways), representing three thresholds of
614 missing genotype data across individuals: 25%, 20%, and 15%. We first imputed the data
615 sets with *snmf* using the optimized settings (identified by minimizing the average cross
616 entropy, with identical optimized parameters for all data sets): alpha=10, and K=1
617 (supplemental Table S8). We modified the default genomic inflation factors to produce p-
618 value distributions that better met the uniform distribution assumption (per Francois et

al., 2016, p-value histograms provided in supplemental Figures S6-S11), providing a balance between true positive detections and false positives driven by population structure and other potential confounders. We did not include other corrections for confounding factors in the GEA since population structure is low across the study area (e.g., Table 3), and dbRDA is robust to isolation-by-distance demographic scenarios (Forester et al., 2018) such as this one. Environmental predictor variables within each region were not highly correlated (i.e., all pairwise correlations were less than |0.8| and all variance inflation factors, which measure multicollinearity, were less than five, where values >10 can be problematic), and none of the retained predictors were highly correlated with either latitude or longitude (supplemental Tables S9, S10). For the outer strata and inland waterways, we identified as candidates those microhaplotypes that were detected in at least two of the three thresholded data sets. This produced 22 candidates for the outer coastal region and six candidates for the inland waterways region. Candidate microhaplotypes for the outer coastal region showed the strongest relationships with the mean and standard deviation of daily sea surface temperature (sst.mn and sst.SD), and the mean of daily sea surface height (ssh.mn). Triplots of the dbRDA results illustrated relationships among individual's multilocus genotypes, the candidate microhaplotypes, and environmental predictors. For example, the southernmost Morro Bay individuals, located in the ordination space as a function of their genotypes at the candidate loci, showed strong relationships with increasing ssh.mn and sst.mn and decreasing ssh.SD, indicating potential adaptation to local environmental conditions (Figure 6A). The smaller inland waterways data set showed some differentiation between the Neah Bay and San Juan Islands individuals based on sea surface temperature, with the Puget Sound individuals showing no relationships, possibly due to their most recent re-colonization (Figure 6B). Because loci under selection could affect population structure, we confirmed that identification of divergence between adjacent a priori strata were consistent with and without the loci identified by dbRDA analysis (supplemental Table S7). Full dbRDA triplots and microhaplotype biplots are provided in supplemental Figures S12-S15 for the outer coastal data and supplemental Figures S16-S19 for the inner waterways.

Discussion

The North Pacific harbor porpoise is one of several geographically and genetically described subspecies of harbor porpoise, some of which consist of multiple ecotypes (Ben Chehida et al., 2020; Fontaine, 2016; Fontaine et al., 2014; Fontaine et al., 2012). Studies of the North Atlantic subspecies have suggested both historical biogeographic processes (Fontaine, Baird, et al., 2007; Fontaine et al., 2014) and ecological processes (Fontaine et al., 2017; Fontaine, Tolley, et al., 2007; Lah et al., 2016) resulting in divergent populations and limited dispersal and introgression between adjacent types. In the North Pacific, evidence of structure based on both biogeographic (Taguchi, Chivers, Rosel, Matsuishi, & Abe, 2010) and ecological/spatial divergence (Chivers et al., 2002; Chivers et al., 2007; Crossman et al., 2014) has been more limited, but suggests that similar processes may have acted across the ranges of subspecies in both ocean basins.

Previous genetic studies of North Pacific harbor porpoise have been limited by sample availability, sample quality, and low power to detect population genetic differences within this nearly continuously distributed coastal species. We have developed a set of SNP and microhaplotype loci that provide sufficient genetic power to detect low levels of population structure, while allowing us to make use of poor-quality tissue samples available from beach-cast and fishery bycatch carcasses. We used hierarchical partitioning to explore evidence of population structure across the range, and to infer potential barriers to geneflow. Correlation of remotely-sensed environmental variables with genetic patterns identified factors potentially influencing local adaptation. Our results indicate that North Pacific harbor porpoises exhibit genetic discontinuity and limited dispersal that may be associated with habitat variability and local adaptation.

Previous evidence indicated intrageneric hybridization between the more pelagic Dall's porpoise and the coastal harbor porpoise, based on morphology and genetic analysis of eight microsatellite loci (Crossman et al., 2014). While the previous genetic analyses suggested bi-directional hybridization and introgression, the more powerful dataset used here indicates that many of the putative hybrids (based on genetics) could be assigned

with >98% probability to one or the other species (N=5 *P. phocoena*; N=2 *P. dalli*), or as F1 hybrids (N=5) between male harbor porpoise and female Dall's porpoise. Sightings of putative hybrids in the wild have also all been associated with Dall's porpoises (Baird et al., 1998). Harbor porpoise males have disproportionately large testes and have been observed to exhibit forceful and fast ambush mating (Keener, Webber, Szczepaniak, Markowitz, & Orbach, 2018), suggesting a greater reliance on sperm competition rather than mate choice in male harbor porpoise. This mating strategy might result in occasional intergeneric hybrids with Dall's porpoise in areas where their ranges overlap (Baird et al., 1998). Although the lack of individuals exhibiting intermediate probabilities of assignment to both species (between 50% and 100%) suggests hybrids are usually infertile, there have been sightings of putative hybrid females with neonatal calves (Willis et al., 2004).

Within harbor porpoises, our results are consistent with isolation by distance along a mostly linear range from southern California to western Vancouver Island, and through the inland waterways, but also suggest some regions of higher genetic divergence than expected based on geographic distance alone. The two largest population splits are between Morro and Monterey Bays in the south, and between the Strait of Georgia and San Juan Islands in the north, where multiple methods suggest divergence in both mitochondrial DNA and nuclear markers. This break between the Strait of Georgia and San Juan Islands, as well as between the San Juan Islands and the outer Strait of Juan de Fuca, is consistent with the limited movements of tagged harbor porpoises in this area (Hanson, 2007). Another population break is suggested between the Monterey Bay and San Francisco sample sets, based on the Monmonier analysis of the connection network (Figure 4), where F_{ST} divergence is significant for nuclear loci but not for mtDNA.

Although the Morro Bay population is designated as a separate management stock based on a hiatus in distribution at the Big Sur coast (Carretta et al., 2019), our results provide the first evidence for genetic differences. The most common mtDNA haplotype (CR01) was found in every *a priori* geographic group except Morro Bay, and the most common haplotype in Morro Bay (CR02) was found in 86% of the Morro Bay samples, but less

712 than 33% of any other population (range 0 – 33%). Two (CR30, CR42) of the three other
713 haplotypes found in Morro Bay were unique to that population, and differed from CR02
714 by only one nucleotide change. The third haplotype (CR03) was distributed across most
715 of the range, but represents a common haplotype that is more similar to CR02 (3bp
716 different) than to the cluster including CR01 (7bp different) and the majority of the other
717 haplotypes distributed throughout the rest of the range (see haplotype network,
718 supplemental figure S5). This suggests long-term isolation of the southernmost
719 population, as well as a persistently small population size, or severe or recurrent
720 bottlenecks. Annual estimates of tens to hundreds of harbor porpoises were killed in
721 gillnet entanglements between approximately 1960 and 2001, resulting in a reduction of
722 the population by 30-97% (Barlow & Hanan, 1995). The population has since begun
723 recovering, from a low of 560 in 1990 to 4255 in 2012 (Carretta et al., 2019; Forney et
724 al., in press). Our results suggest that recovery has been due to internal recruitment rather
725 than migration from the larger Monterey Bay population to the north.
726
727 The northernmost strata were the most differentiated, with high mtDNA divergence
728 metrics and the highest frequencies of the most common control region haplotype (CR01;
729 66% W. Vancouver Is., 63% Strait of Georgia). The frequency of this haplotype ranged
730 from zero to 50% in other strata. The BC stratum (excluding Strait of Georgia samples)
731 spanned a large geographic range with few samples (N=5), and included two private
732 mtDNA haplotypes, both found in two samples. These suggest different haplotype
733 composition in this region, but we cannot rule out sampling of related individuals or
734 isolated regional populations, as the sample size is small and shared haplotypes were
735 found in samples that were collected close in space and time. Combined, these data
736 suggest that there is a haplotype gradient between the northern and southern portions of
737 the range, possibly representing interglacial range expansion (Taguchi et al., 2010) or the
738 presence of a historical phylogeographic separation that has more recently re-connected,
739 as has been suggested for populations in the eastern Atlantic Ocean (Fontaine, Baird, et
740 al., 2007; Fontaine et al., 2014).
741

742 Harbor porpoise habitat in the eastern North Pacific includes the California Current
743 System (CCS), recognized as one of the most productive marine ecosystems on the
744 planet, but spans topographically, oceanographically and temporally complex regions
745 from temperate to Arctic waters. Aside from preference for coastal waters less than 200
746 m deep, little is known about factors influencing suitable habitat for Pacific harbor
747 porpoises, which vary in population density and abundance along the west coast and
748 inland waters of North America. The remotely sensed and modeled oceanographic
749 variables available throughout most of this range (upwelling index, mixed-layer depth,
750 sea surface temperature and sea surface height) are all proxies for habitat variability with
751 complex relationships to wind, currents, topology, isocline depth, productivity, fresh-
752 water input and seasonal and interannual effects (Castelao & Luo, 2018; Hickey et al.,
753 2016; Venegas et al., 2008). Our genotype-environment association analyses provide
754 initial evidence for local adaptation to environmental variability across the study range.
755 Given the limited scale of genomic sampling in this study these findings should be seen
756 as preliminary, providing a basis for future investigation of local adaptation across the
757 complex seascape environment inhabited by harbor porpoise (see, e.g., Fontaine et al.,
758 2017; Fontaine, Tolley, et al., 2007; Nielsen et al., 2018).
759
760 An overall correlation with latitude, which might be expected across a large latitudinal
761 range, appears to strengthen in the regions south of Oregon. In central Oregon, there is an
762 oceanographic shift around Cape Blanco, where the coastal upwelling jet separates from
763 the coast (~15-30 km offshore), becoming an oceanic jet (>100 km offshore) (Castelao &
764 Luo, 2018). Populations in the northern half of the coastal range are associated with
765 several environmental variables (e.g., mean and SD of the mixed layer depth, and high
766 variation in both sst and ssh), while those in the central and southern portions are
767 associated with others (e.g., high mean and low variation in ssh) (Figure 6). There is also
768 evidence of population structure across that region, with significant nuclear genetic
769 differentiation among populations from southern Washington to northern California
770 (Table 3). There was a particularly strong correlation between increased mean ssh and
771 decreased ssh variability in the southernmost Morro Bay population, which is also the
772 most genetically distinct, suggesting possible local adaptation linked to oceanographic

773 processes (e.g., thermocline and/or upwelling). While these links are indirect, they
774 provide the first evidence of local adaptation in addition to demographic independence
775 among harbor porpoise populations along the west coast of North America. The Morro
776 Bay stock was previously recognized based only on a distribution hiatus and evidence of
777 historical population decline due to extensive fishery bycatch (Barlow & Hanan, 1995),
778 as well as blubber pollutant ratios that differed from other areas (Calambokidis &
779 Barlow, 1991). It is potentially subject to offshore energy production disturbance (Forney
780 et al., 2017), and is also at the southern edge of the species' geographic range, and most
781 likely to be impacted by climate change (Learmonth et al., 2006; Ruiz-Cooley et al.,
782 2017). Our results provide strong support for continued management of this population as
783 a separate management stock, especially in light of potential impacts of coastal
784 development and climate change.
785
786 Harbor porpoise populations along the west coast of North America have historically
787 experienced substantial fisheries bycatch in portions of the range (Barlow & Hanan,
788 1995), and multiple lines of evidence indicate that there is limited dispersal among
789 regions. Within the U.S., this led to identification of five coastal management stocks
790 between southern California and the northern U.S. border (coinciding with four of the
791 geographic strata in this study, plus the combined S. Oregon and N. California strata),
792 and one stock in the inland waterways in Washington (Carretta et al., 2019), with stock
793 boundaries placed to coincide with areas of lower density. The genetic results presented
794 here provide additional evidence of limited movement among the currently defined
795 management stocks, as well as between some geographically defined strata within
796 existing stocks, consistent with previous studies of genetics and contaminant levels
797 (Calambokidis & Barlow, 1991; Chivers et al., 2002), and limited movements of tagged
798 porpoises in the inland waterways of Washington (Hanson, 2007). In particular, sPCA
799 and F_{ST} analyses of nuclear data show separation of the N. California and S. Oregon
800 strata (sPCA randtest $p = 0.017$) with a small but significant divergence (F_{ST}=0.008) in
801 nuclear allele frequencies (Table 3, Figure 3D, supplemental Figure S3D). We suggest
802 that the lack of statistically significant difference in mtDNA is likely a result of low
803 statistical power due to small sample size in N. California (n=11) rather than results that

804 contradict the finding of differences between these strata based on nuclear markers.
805 Placement of a potential stock boundary as indicated in Figure 5 is suggested by the drop
806 in density in a region where there is a gap in our sample distribution, though the location
807 of the density minimum in Figure 5 varies through time (supplemental Figure S20), either
808 due to sampling differences among surveys or small north-south shifts of populations
809 across years.
810
811 Genomic methods are evolving rapidly, increasing both the number and variety of genetic
812 markers that can be used to understand evolution, population structure, historical
813 demography, and ecological adaptations (e.g., Tan et al., 2019). This study of harbor
814 porpoise population structure has built on previous research by expanding the number
815 and geographic distribution of samples and the number and type of genetic markers. We
816 also apply new analytical methods to infer patterns of spatial genetic variation, and
817 investigate their correlation with environmental variables that may drive local adaptation.
818 Genetic analyses have been identified as potentially high value for stock delineation
819 (Martien et al., 2019) and these results will be useful for harbor porpoise management
820 stock structure refinement. Nevertheless, inference remains limited by the uneven
821 distribution of opportunistic samples, potential shifts in populations during multiple
822 decades of sample accumulation, and limited availability of environmental variables
823 across the spatial and temporal scale of this study. Additional studies using genome-wide
824 genetic data from across the range are needed to more fully understand habitat use and
825 local adaptation in the eastern North Pacific, and their importance for harbor porpoise
826 management and conservation.
827
828
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Data accessibility

- Mitochondrial DNA haplotype sequences used in this study are complete or partial sequences of sequences previously available in Genbank (Accession numbers provided in Supplemental Table S2).

Nuclear genotypes and environmental data associated with the samples: Dryad doi: https://doi.org/10.5061/dryad.4tmpg4f6v

Author contributions

P.A.M. K.P. and B.L.T designed the research project, and P.A.M and K.P. performed analyses and wrote the paper. K.A.F., B.R.F. contributed environmental data and analyses, and contributed to writing the paper. B.L.H.-H., K.M.R., C.A.C., L.G.B.-L., C.S., T.H. and M.C.F. contributed samples and/or genetic data used for analyses. All authors contributed to preparation of the manuscript.

Figure 1. Sample distribution colored by *a priori* geographic strata used for analyses. Current U.S. stocks are shown with horizontal lines demarcating the boundaries. The Washington Inland Waters stock is shown with the shaded polygon.

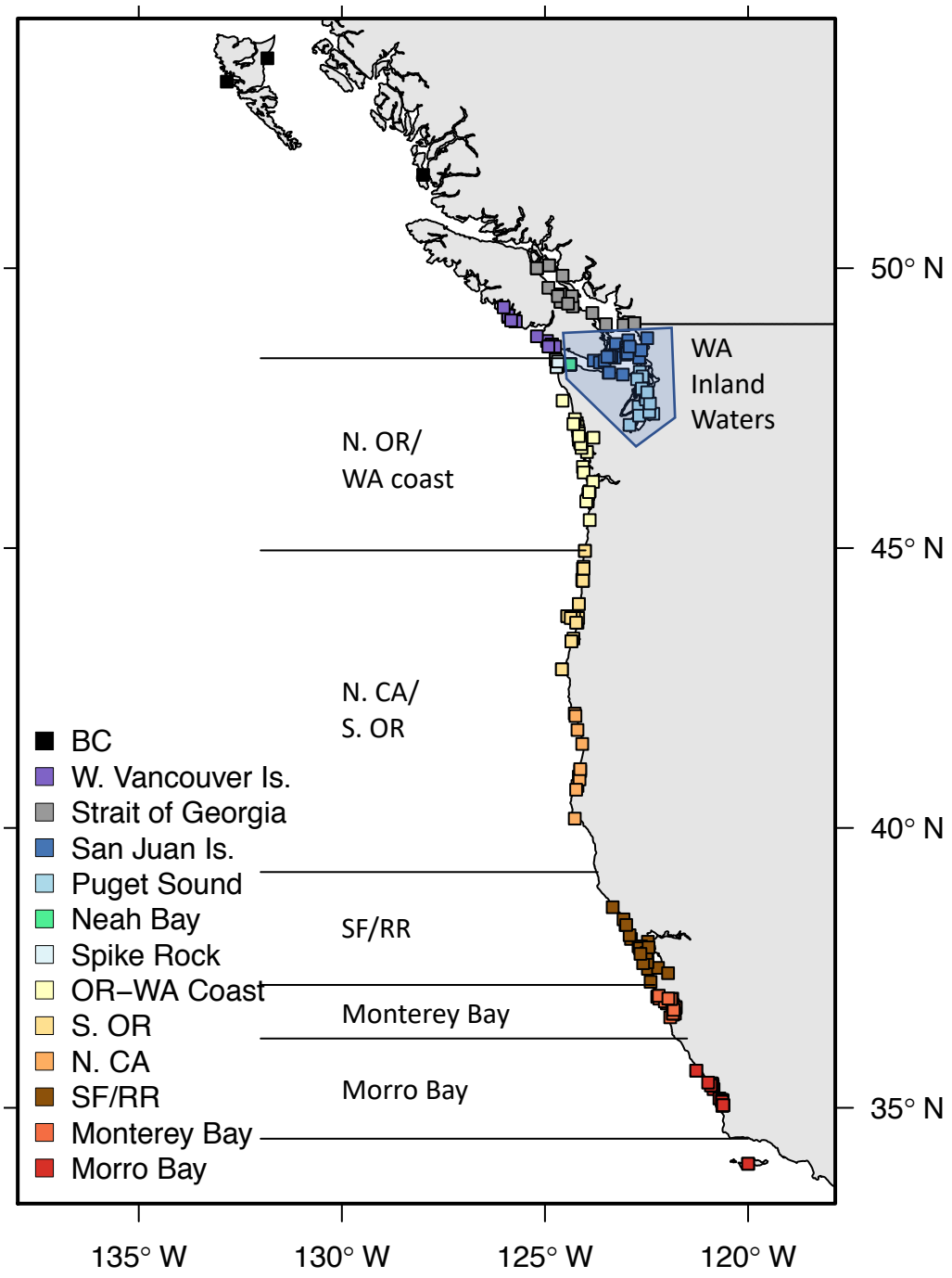
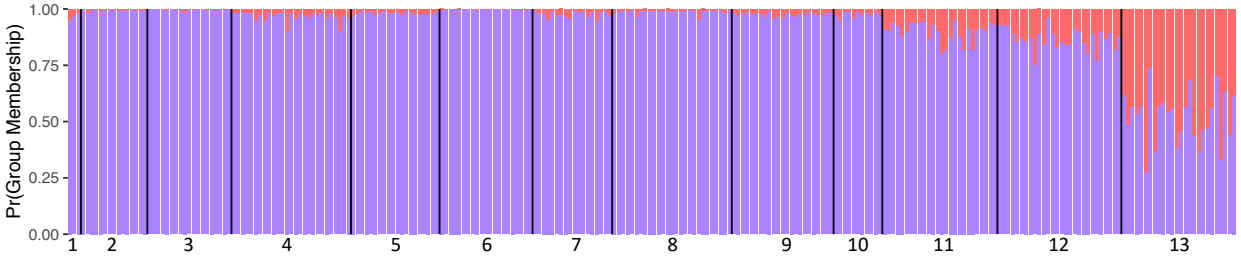


Figure 2. STRUCTURE plots for K = 2 and K = 3 based 290 loci and 264 samples (unrelated) with 10 replicates combined with CLUMPP. Samples assigned to *a priori* geographic strata are sorted by decreasing latitude in the bar plot from left to right. 1 = BC; 2 = W. Vancouver Is.; 3 = Strait of Georgia; 4 = San Juan Is.; 5 = Puget Sound; 6 = Neah Bay; 7 = Spike Rock; 8 = OR-WA coast; 9 = S. OR; 10 = N. CA; 11 = SF/RR; 12 = Monterey Bay; 13 = Morro Bay.

K = 2 (ΔK = 59.1)



K = 3 (ΔK = 0.9)

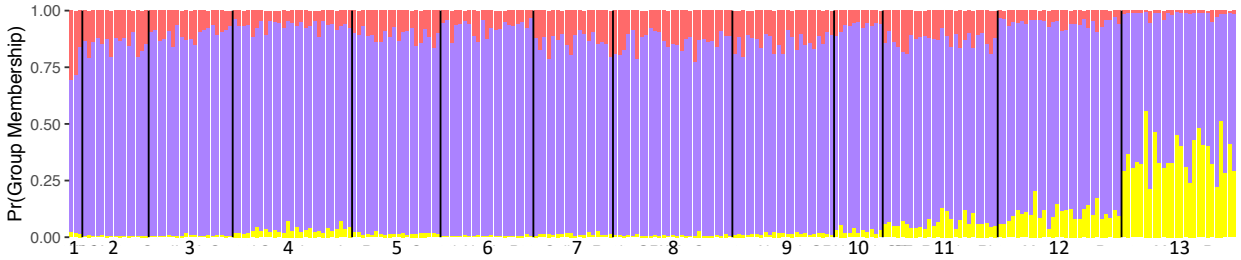


Figure 3. sPCA three-dimensional plot of the first three lagged scores of spatial PCs (sPC) for samples from A) all strata and samples (290 loci), and two-dimensional plots of the first two sPCs for geographical subsets of the data from B) outer coast strata (all strata except BC and those in (C); 290 loci), C) inland waters of Washington and British Columbia (Strait of Georgia, San Juan Is., Puget Sound, Neah Bay; 289 loci), D) central California / southern Oregon (S. OR, N. CA, SF/RR, Monterey Bay 288 loci). Insert bar-charts show the eigenvalues. The ovals represent ellipses of dispersion. Colors correspond to *a priori* strata, with sample numbers per stratum as in Table 3. Different numbers of loci are due to removal of monomorphic loci in sample subsets.

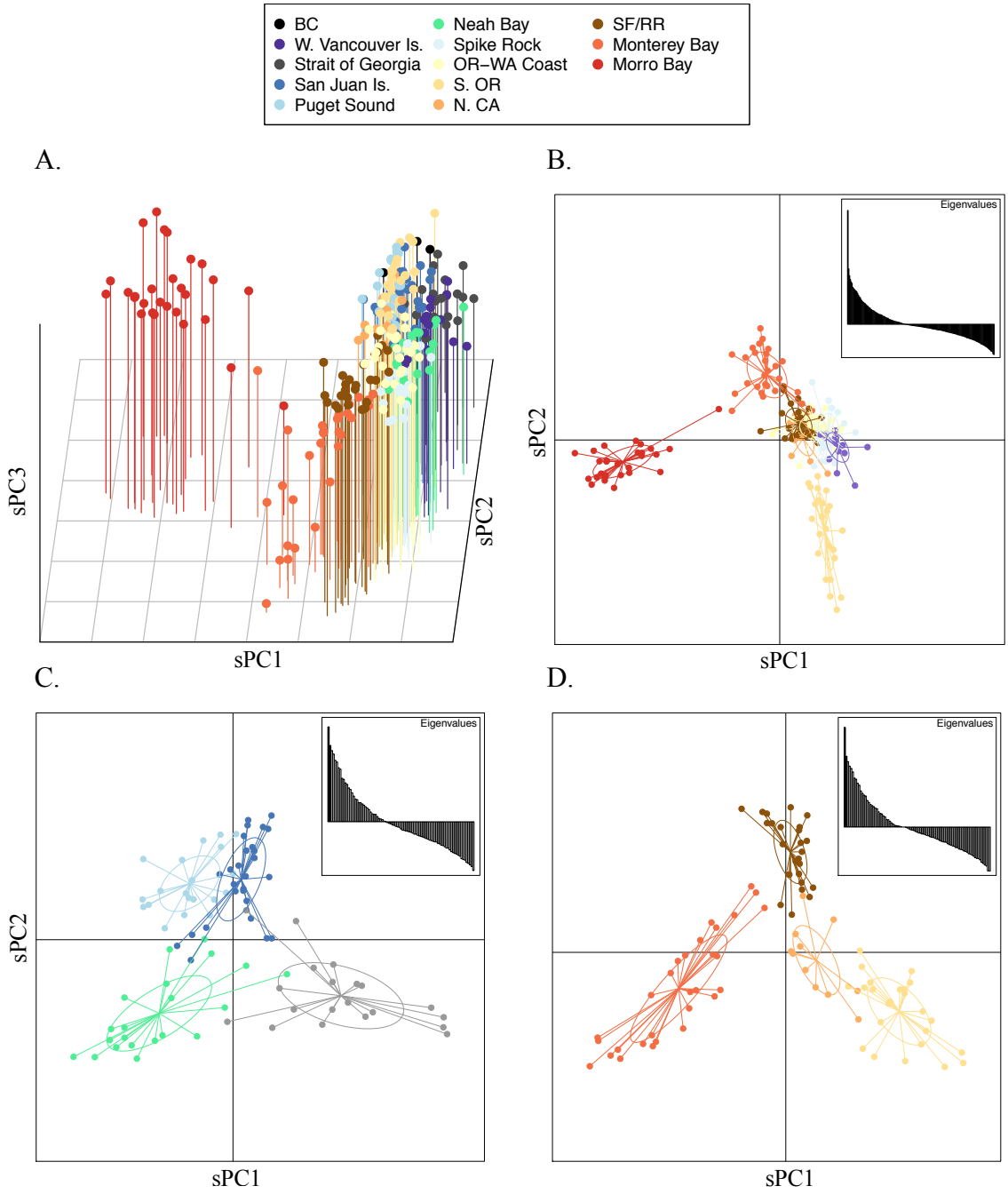


Figure 4. A) Gabriel connection network from a Monmonier analysis of samples (adjusted to prevent sample location overlap) with local nuclear genetic differences above the threshold value indicated by thicker blue lines and arrows between geographic distance edges for A) the outer coastal samples, with subsets of samples shown for localized thresholds, and B) inland waterways of Washington and British Columbia. Inset maps are for orientation of sample networks. Thickness of the lines and arrows is proportional to the level of genetic difference above the threshold level. Colors correspond to *a priori* strata as in Figure 1.

A B

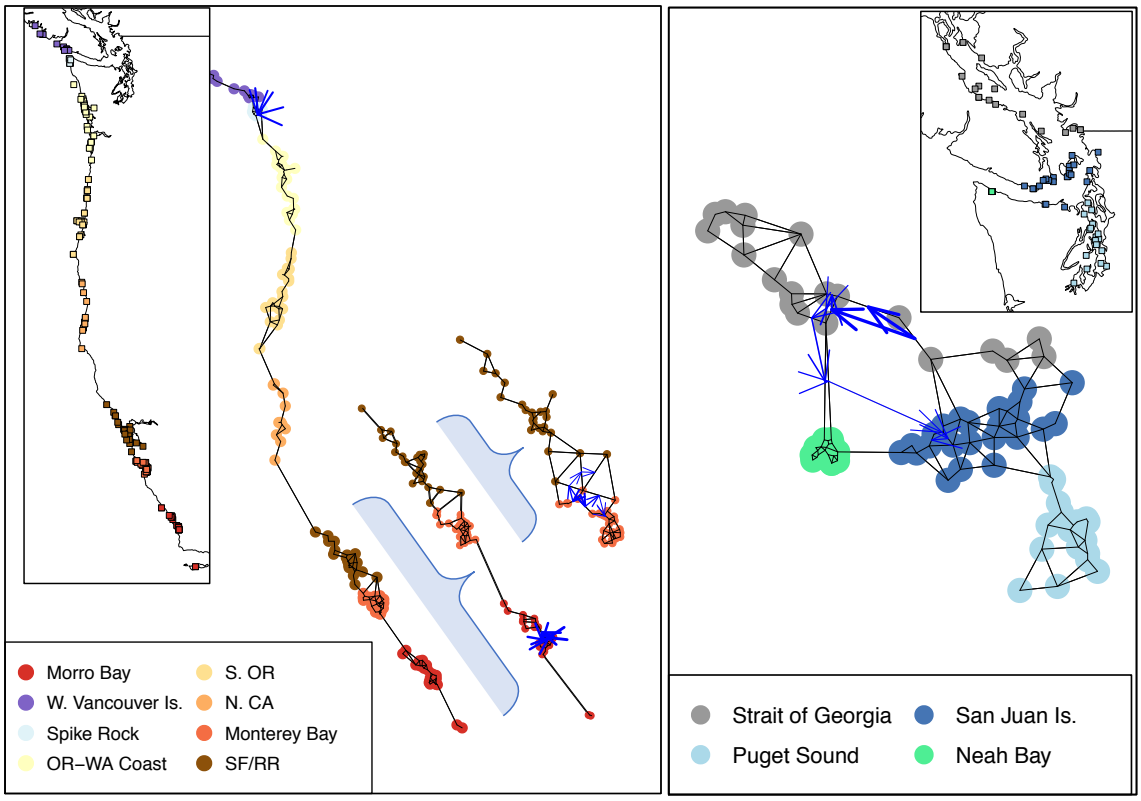


Figure 5. Pie charts represent frequencies of haplotypes represented by ≥ 5 samples in the stratum, plus two haplotypes (CR05, CR35) that were found only in the BC strata, in two samples each (out of 5 total). Relative porpoise density estimates (porpoise per kilometer surveyed) are plotted by latitude, averaged across multiple survey periods (blue line). The current U.S. stocks are named and boundaries are represented by horizontal black lines (as in Figure 1). The proposed boundary location for splitting the Northern California/Southern Oregon stock into two stocks is shown with a dashed red line.

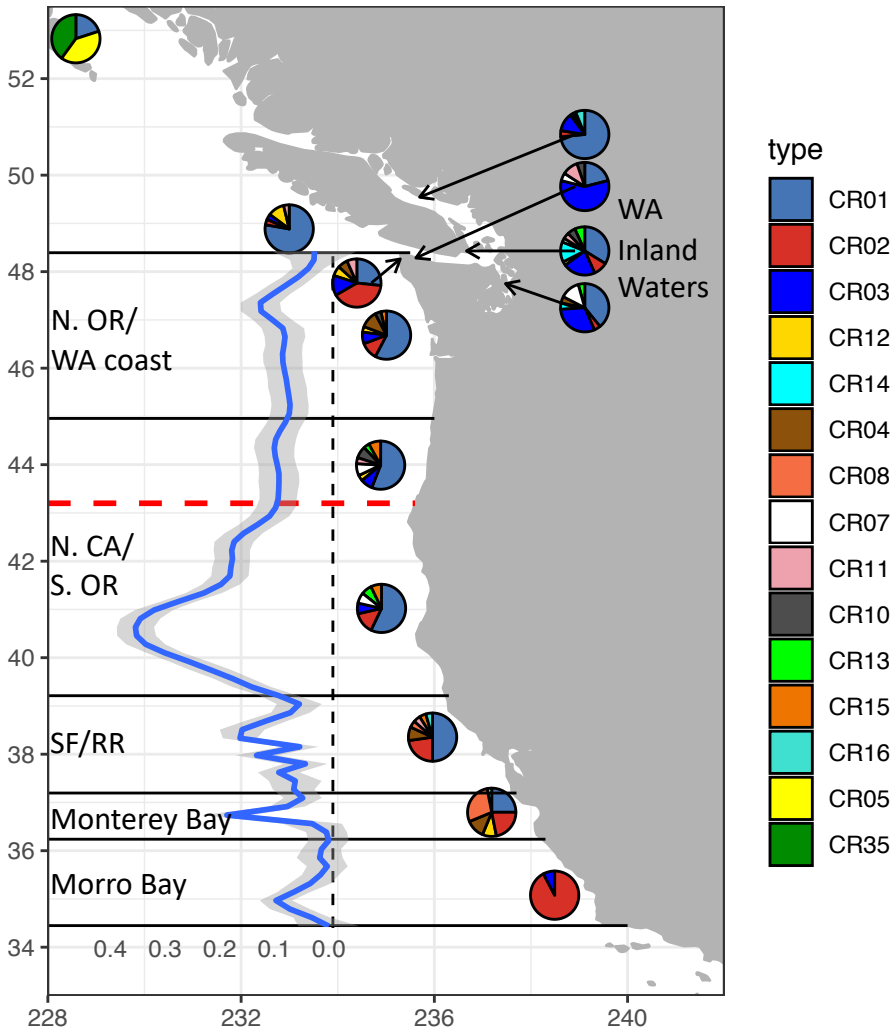
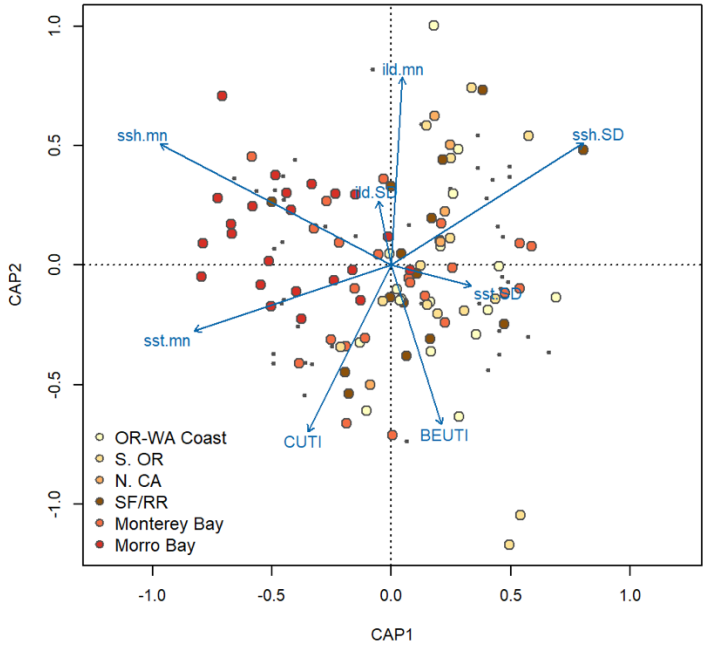


Figure 6. Distance-based redundancy analysis triplot of candidate microhaplotypes under selection for A) the outer coastal strata and B) inland waterways strata, showing individuals as colored circles, microhaplotype alleles as grey points, and environmental predictors (see Table 1 for abbreviations) as blue arrows. The location of individuals in the ordination space reflects their relationship with the environmental variables based on their multi-locus genotypes at the candidate microhaplotype markers.

A)



B)

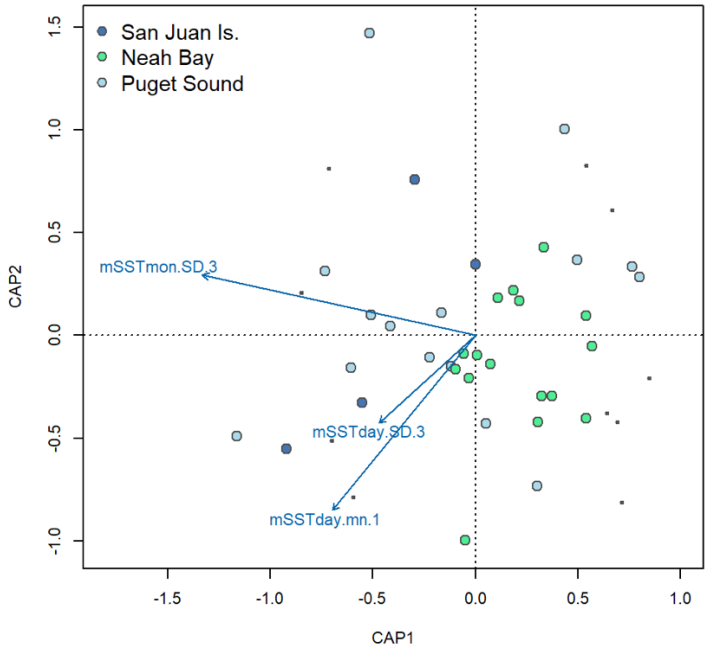


Table 1. Environmental predictors for distance-based redundancy analysis. SD = standard deviation.

Analysis	Environmental predictor	Abbreviation	Predictor resolution	Source
Outer coastal populations	Mean daily sea surface temperate	sst.mn	Averaged over 0.1 degrees around sample location	Regional Ocean Modeling System ROMS/CCSRA 31-year reanalysis (1991-2010) or Near Real Time (2011+), from the U.C. Santa Cruz Ocean Modeling and Data Assimilation group; http://oceanmodeling.ucsc.edu/; (Moore et al., 2011)
	SD of daily sea surface temperate	sst.SD	Calculated within 0.3 degrees around sample location	
	Mean daily sea surface height	ssh.mn	Averaged over 0.1 degrees around sample location	
	SD of daily sea surface height	ssh.SD	Calculated within 0.3 degrees around sample location	
	Mean daily mixed layer depth (depth at which the temperature is -0.5°C from surface temperature)	ild.mn	Averaged over 0.1 degrees around sample location	
	SD of daily mixed layer depth (depth at which the temperature is -0.5°C from surface temperature)	ild.SD	Calculated within 0.3 degrees around sample location	
	Coastal Upwelling Transport Index	CUTI	Calculated every 1 degree latitude along West Coast from 31N-47N	http://mjacox.com/upwelling-indices/ (Jacox et al., 2018)
	Biologically Effective Upwelling Transport Index	BEUTI	Calculated every 1 degree latitude along West Coast from 31N-47N	
Inland waterways populations	Mean daily sea surface temperate (comparable to sst.mn above)	mSSTday.mn0.1	Averaged over 0.1 degrees around sample location	Multispectral Ultra-high Resolution Sea Surface Temperature (murSST); downloaded from NOAA/ERDDAP (Simons, R. A. 2015. Environmental Research Division Data Access Program ERDDAP. https://upwell.pfeg.noaa.gov/erddap. Data set names: jplMURSST41SST (daily) and jplMURSST41mday (monthly)
	SD of daily sea surface temperature (comparable to sst.SD above)	mSSTday.SD 0.3	Calculated within 0.3 degrees around sample location	
	Mean monthly sea surface temperature	mSSTmon.mn 0.1	Averaged over 0.1 degrees around sample location	
	SD of monthly sea surface temperature	mSSTmon.SD 0.3	Calculated within 0.3 degrees around sample location	

Table 2. SNP and microhaplotype genotype summary information, based on 292 loci.

Species	Samples	Hexp.	Hobs.	Monomorphic
P. phocoena	281	0.366	0.356	2
P. dalli	11	0.144	0.145	177
hybrid	5	0.479	0.324	64

Table 3. Pairwise F_{ST} for nuclear loci (lower left) and mtDNA haplotypes (upper right). Statistically significant values (p<0.05) are in bold. Sample sizes each population is shown in parentheses for nuclear loci on the left, and mtDNA on the top. The p-values for adjacent strata pairwise comparisons are provided in supplemental Table S9.

	BC (5)	W. Vanc. Is (29)	Strait of Georgia (55)	San Juan Is (92)	Puget Sound (24)	Neah Bay (20)	Spike Rock (18)	ORWA Coast (30)	SoOR (29)	NoCal (17)	SF_RR (30)	Monterey Bay (37)	Morro Bay (28)
BC (3)	--	**0.223**	**0.249**	**0.097**	**0.147**	**0.251**	**0.127**	0.151	**0.140**	0.126	**0.108**	**0.125**	**0.604**
W. Vancouver Is (15)	-0.003	--	-0.005	**0.088**	**0.076**	**0.247**	**0.128**	0.002	0.005	0.004	**0.046**	**0.130**	**0.542**
Strait of Georgia (20)	-0.006	0.005	--	**0.095**	**0.066**	**0.232**	**0.152**	0.011	0.017	0.012	**0.064**	**0.159**	**0.526**
San Juan Is (27)	0.005	0.000	**0.005**	--	0.008	**0.066**	0.023	**0.043**	**0.037**	0.021	**0.031**	**0.059**	**0.307**
Puget Sound (20)	0.003	0.003	**0.009**	0.001	--	0.039	0.057	0.029	0.020	0.009	**0.046**	**0.093**	**0.454**
Neah Bay (21)	**0.015**	0.000	**0.010**	0.000	0.002	--	**0.141**	**0.176**	**0.157**	**0.160**	**0.174**	**0.192**	**0.531**
Spike Rock (19)	0.002	-0.004	**0.007**	0.002	0.002	-0.001	--	**0.049**	**0.083**	0.033	0.005	0.024	**0.233**
ORWA Coast (28)	0.015	-0.001	**0.014**	**0.004**	**0.005**	**0.004**	-0.001	--	-0.006	-0.023	-0.005	**0.067**	**0.440**
SoOR (24)	0.009	0.002	**0.011**	0.002	-0.001	**0.004**	0.001	**0.006**	--	-0.027	0.019	**0.090**	**0.480**
NoCal (11)	0.014	0.003	**0.015**	0.001	0.005	0.003	0.004	0.002	**0.008**	--	-0.012	**0.059**	**0.448**
SF_RR(26)	0.012	0.004	**0.015**	**0.005**	0.004	**0.005**	-0.002	**0.004**	0.002	0.004	--	0.023	**0.347**
Monterey Bay (29)	**0.021**	**0.006**	**0.016**	**0.007**	**0.011**	**0.008**	0.001	**0.006**	**0.012**	**0.007**	**0.005**	--	**0.310**
Morro Bay (28)	**0.027**	**0.028**	**0.036**	**0.022**	**0.024**	**0.028**	**0.023**	**0.026**	**0.025**	**0.024**	**0.020**	**0.020**	--

Table 4. dbRDA sample information for three data sets filtered for different levels of missing genotypes. Rows in italics report the number of retained individuals in each set of populations. GIF = genomic inflation factor; FDR = false discovery rate.

	Outer coastal populations			Inland waterways populations			
Amount of missing data	25%	20%	15%	25%	20%	15%	
Microhaplotypes retained	280	280	280	274	274	274	
Individuals retained	98	96	92	36	36	31	
OR-WA Coast	*17*	*17*	*14*	*4*	*4*	*4*	*San Juan Is.*
S. OR	*14*	*14*	*14*	*16*	*16*	*14*	*Neah Bay*
N. CA	*5*	*5*	*5*	*16*	*16*	*13*	*Puget Sound*
SF/RR	*16*	*14*	*13*	-	-	-	
Monterey Bay	*23*	*23*	*23*	-	-	-	
Morro Bay	*23*	*23*	*23*	-	-	-	
Modified GIF	1.19	1.20	1.20	1.14	1.14	1.18	
Candidates at FDR=0.1	25	22	4	6	6	9	