Population structure and mechanisms of gene flow within island-associated false killer whales (*Pseudorca crassidens*) around the Hawaiian Archipelago

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Abstract

We use mitochondrial sequence (mtDNA) data and data from 16 nuclear microsatellite markers (nucDNA) to examine genetic structuring of false killer whales (*Pseudorca crassidens*) within the Hawaiian Archipelago. We compare samples collected around the main Hawaiian Islands (MHI) from members of the Hawaiian Insular population to samples from individuals that have been hypothesized to represent a previously undocumented island-associated population in the Northwest Hawaiian Islands (NWHI). We find significant genetic differentiation between the groups in both mtDNA and nucDNA, indicating that they represent separate populations between which gene flow is limited. We use parentage analyses to evaluate social structure within the MHI. Our results indicate that both males and females exhibit strong fidelity to natal social groups and that mating occurs both within and between social groups. Such a mating system could result in inbreeding depression, further imperiling a population that we estimate has an effective population size of only 50.5 individuals. Our finding of male-mediated gene flow but no sex-biased dispersal provides important insight into the mechanisms of gene flow in this species.

Introduction

The Hawaiian Insular population of false killer whales (*Pseudorca crassidens*) has been the subject of intense scientific scrutiny in recent years. A long-term photo-identification study has revealed it to be small population of approximately 150 animals that is restricted to the waters around the main Hawaiian Islands (Baird *et al.* 2008, Baird *et al.* 2010). Though satellite data indicates limited range overlap between Hawaiian Insular false killer whales and pelagic false killer whales (Baird *et al.* 2010), insular and pelagic whales have never been documented in association with each other. Genetic studies have revealed strong differentiation between the insular population and the rest of the North Pacific, with nearly every member of the insular population possessing one of two closely related mitochondrial haplotypes that are unique to the insular population (Chivers *et al.* 2007, Chivers *et al.* 2010). The insular population is also significantly differentiated at nuclear microsatellite loci, though the estimates of differentiation are much lower than for mitochondrial DNA. The discrepancy in differentiation between the two markers could be an artifact of the high diversity of the nuclear markers, or could indicate that nuclear differentiation is being limited by either male-mediated gene flow or male-biased dispersal (Chivers *et al.* 2010).

The Hawaiian Insular population has only been documented around the main Hawaiian Islands. However, during the 2010 HICEAS II research survey there were

sightings of false killer whales near several of the Northwest Hawaiian Islands (NWHI). As during the 2002 HICEAS cruise, few false killer whale sightings occurred in pelagic waters. Satellite tracking data from an animal tagged near Nīhoa showed the animal traveling back and forth along the islands and seamounts between Nīhoa and French Frigate Shoals for the entire 52 day duration of the track (Baird *et al.* 2011b). Photographs taken from three encounters involving the tagged animal revealed that almost half of the animals were present in two or more of the encounters (Baird *et al.* 2011b). Photo-identification data also revealed matches between these three encounters and two groups encountered off of Kauaʻi in 2008. The Kauaʻi animals have not been seen in association with any members of the Hawaiian Insular population, and so are not considered part of that population (Baird 2009), though it is not known whether they represent rarely encountered members of the insular population, the pelagic population, or a third, yet undefined population (Oleson *et al.* 2010). The fact that the NWHI animals match photographically to animals seen off Kauaʻi two years earlier suggests that they are multi-year residents and that their range overlaps, at least spatially and perhaps temporally, with that of the Hawaiian Insular population (Baird 2009).

In addition to new data from the NWHI, recent additions to the Hawaiian Insular photo-identification catalog and new social network analyses revealed the existence of three large, distinct social groups within the population (Baird *et al.* 2011a). Analysis of sighting histories revealed differences in habitat use among the three clusters, with the sightings of individuals from cluster 2 occurring off the island of Hawaiʻi more frequently than expected, given the overall distribution of sightings. Based on satellite tagging data, Baird et al. (2011a) showed that individuals from cluster 1 use significantly shallower depths (median = 608m) than individuals from cluster 3 (median = 1052m). No tags were deployed on individuals from cluster 2.

We examine genetic structuring of false killer whales across different spatial scales within the Hawaiian Archipelago using data from both mitochondrial (mtDNA) and nuclear microsatellite (nucDNA) markers. We compare the NWHI island-associated individuals to the Hawaiian Insular population to determine whether the NWHI animals represent a demographically independent population of island-associated false killer whales or if they are an extension of the Hawaiian Insular population. We use parentage analysis to determine whether there is a familial basis for the social clusters within the Hawaiian Insular population and, if so, what the mechanism of gene flow is between the clusters. These analyses will improve our understanding of the rates and mechanism of gene flow among clusters within the Hawaiian Insular population and provide an appropriate context for interpreting the levels of differentiation between the Hawaiian Insular population and the NWHI island-associated animals.

Methods
Sample collection
Our study includes biopsy samples collected from two distinct areas of the Hawaiian Archipelago: the Main Hawaiian Islands (MHI) and the eastern portion of the Northwest Hawaiian Islands (NWHI; Figure 1). Samples from the MHI were collected between 2000 and 2010. Most of these samples were collected as part of the photo identification study conducted by Baird et al. (2008), while the remainder were collected opportunistically by

other researchers. All samples in our MHI stratum were collected from animals that have been identified as members of the Hawaiian insular population in analyses of photo-identification and association pattern data (Baird *et al.* 2008, Baird 2009).
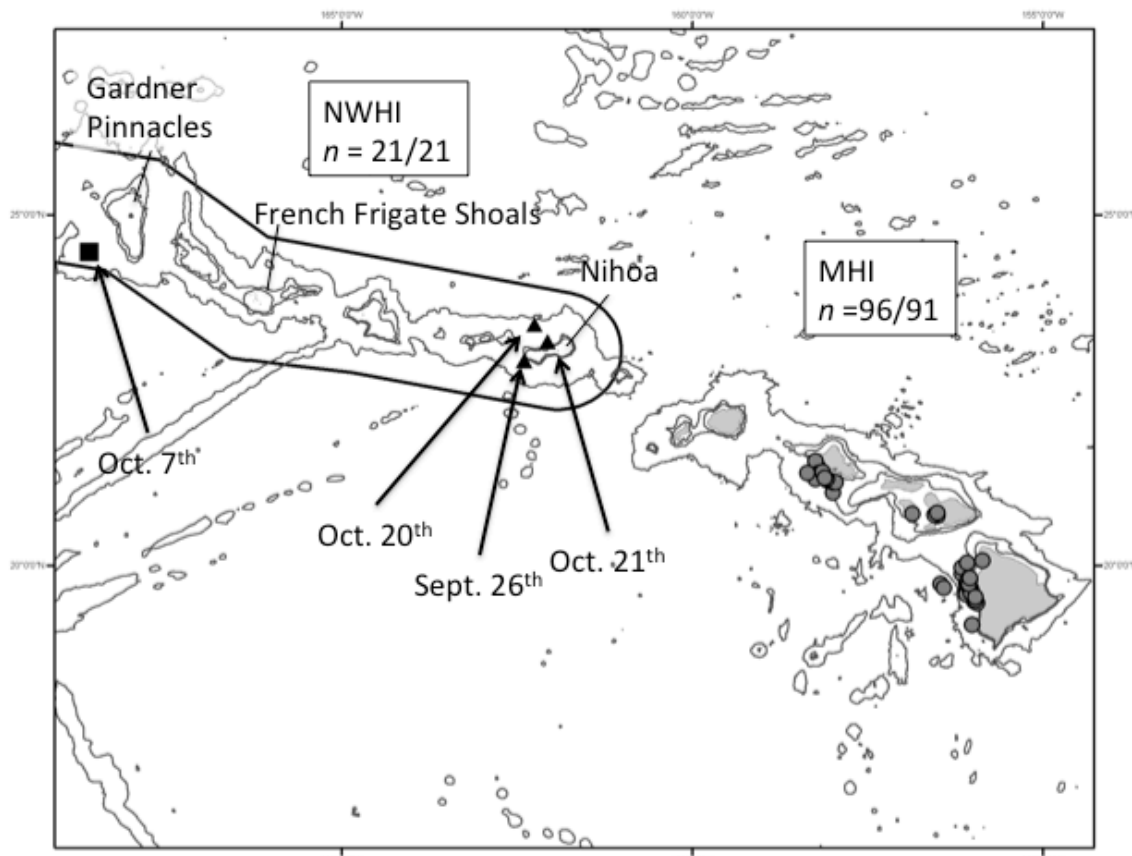

Figure 1. Map showing the locations of samples included in this study. Samples were stratified into main Hawaiian Islands (MHI; gray circles) and Northwest Hawaiian Islands (NWHI; black symbols). Sample sizes (n) are given for the mtDNA/nucDNA data sets. The encounters within the NWHI are labeled according to the date they occurred. The three encounters marked by triangles were identified by Baird et al. (2011b) as representing a previously undocumented island-associated population. The 400m, 1000m, and 4000m depth contours are shown in gray. The black line shows the boundary of the Papahānaumokuākea Marine National Monument

Samples from the NWHI were collected in 2010 during the Southwest Fisheries Science Center (SWFSC) and Pacific Islands Fisheries Science Center (PIFSC) HICEAS II research survey. During the cruise there were eleven encounters with false killer whales within the Hawaiian EEZ, seven of which yielded biopsy samples (Baird *et al.* 2011b). However, we restricted our analyses to the four encounters that occurred in the eastern portion of the Papahānaumokuākea Marine National Monument (henceforth referred to as the Monument). Photo-identification and satellite tagging data suggested that three of these encounters, which occurred Sep. 26, Oct. 20, and Oct. 21, involved animals representing a NWHI island-associated population similar to the Hawaiian Insular

population (Baird *et al.* 2011b). The fourth encounter occurred on Oct. 7, 84 km southwest of Gardner Pinnacles, and did not include any individuals that matched photographically to any other encounter. However, preliminary analyses revealed these animals to possess a haplotype identified by Chivers et al. (2007, 2010) as characteristic of the Hawaiian Insular population. Thus, we included these animals as possible members of an island-associated population. All other samples collected during the HICEAS II cruise, including three collected within the Monument boundary near Midway that possessed pelagic haplotypes, were excluded from our analyses. Analyses involving the full set of HICEAS II samples are presented by Chivers et al. (2011).

   All samples were preserved frozen or in a 20% dimethylsulphoxide solution saturated with NaCl (Amos and Hoelzel 1991, Amos 1997) and archived in the SWFSC Marine Mammal and Turtle Molecular Research Sample Collection (http://swfsc.noaa.gov/PRD-TissueCollection).

mtDNA sequencing
   The 5' end of the hypervariable mtDNA control region was amplified from extracted genomic DNA (sodium chloride protocol: Miller *et al.* 1988, Qiagen DNeasy Blood and Tissue Kit #69505; lithium chloride protocol: Gemmell and Akiyama 1996) using the polymerase chain reaction (PCR) and then sequenced using standard techniques (Saiki *et al.* 1988, Rosel *et al.* 1994). DNA was amplified using a 25µl reaction of 1µl DNA, 18µl of water, 2.5 µl of buffer [10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 µl of 10 mM dNTP], 0.75 µl of each 10 µM primer, and 0.5 units of Taq DNA polymerase. The PCR cycling profile consisted of 90 °C for 2.5 min, followed by 35 cycles of 94 °C for 50 sec, an annealing temperature of 60 °C for 50 sec, and 72 °C for 1.5 min, then a final extension of 72 °C for 5 min. The sequence was generated in two parts. For the first segment, we used primers H16498 (5'-CCTGAAGTAAGAACCAGATG- 3') (Rosel *et al.* 1994) and L15829 (5'-CCTCCCTAAGACTCAAGG- 3') (developed at the SWFSC), and for the second segment, we used primers H497 (5'-AAGGCTAGGACCAAACCT- 3') and L16218 (5'-TGGCCGCTCCATTAGATCACGAGC- 3') (both developed at the SWFSC). The light and heavy strands of the amplified DNA product for each specimen were sequenced independently as mutual controls using standard four-color fluorescent protocols on the Applied Biosystems Inc. (ABI, Foster City, CA) model 377, 3100 and 3730 sequencers. The second segment of approximately 573 base pairs included an approximately 20-base-pair section of overlap with the first 395 base pairs of the control region to ensure all sequences were complete. The final sequences were 947 base pairs long and were aligned using SEQED, version 1.0.3 (ABI) and Sequencher software (versions 4.1 and 4.8; Gene Codes, Ann Arbor, MI).

Microsatellite genotyping and genetic sexing
   Samples were genotyped using microsatellite DNA primers for 16 dinucleotide loci: D12t derived from beluga whales (*Delphinapterus leucas)* (Buchanan *et al.* 1996), EV1t and EV14t derived from sperm whales (*Physeter macrocephalus*) and EV94t derived from humpback whales (*Megaptera novaenglia*) (Valsecchi and Amos 1996), KWM2at, KWM2b, KWM12at derived from killer whales (*Orcinus orca*) (Hoelzel *et al.* 1998), SW19t derived from sperm whales (Richard *et al.* 1996), SL125t and SL1026t derived from spinner dolphins (*Stenella longirostris*) (Galver 2002), and TexVet5t (Rooney *et al.* 1999), Ttr11,

Ttr34, Ttr48, Ttr58 and TtrRC11 (Rosel *et al.* 2005) derived from common bottlenose dolphins (*Tursiops truncatus*). Extracted DNA was amplified using the PCR in 25 μl reactions containing 1 μl (approximately 5-50 ng) genomic DNA, 18.25 μl water, 2.5 μl of buffer [10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$], 0.75 μl of each 10μM primer, 1.5 μl 10mM dNTP and 0.5 units of *Taq* DNA polymerase. The PCR thermal cycling profile for these primers was 90 °C for 2.5 min, followed by 35 cycles of 94 °C for 45 sec, 1 min at annealing temperature, and 72 °C for 1.5 min, then a final extension of 72 °C for 5 min. The optimal annealing temperatures were 48 °C for KWM2at and KWM2b, 50 °C for KWM12at, 54 °C for EV14t, 55 °C for D12t, EV1t, EV94t, SL125t, SL1026t, SW19t, TexVet5t, Ttr11 and TtrRC11, 57 °C for Ttr34 and Ttr48, and 60 °C for Ttr58.

Size and purity of the amplicon were assessed electrophoretically. Genotype data were generated on ABI genetic analyzers (models 3100 and 3730) using a commercial internal lane standard (ROX500®; PE Applied Biosystems Inc.). ABI's GENEMAPPER (version 4.0) software was used to make preliminary allele fragment size 'calls.' GENEMAPPER's calls were reviewed and edited independently by two trained genotypers. The two genotypers compared their calls and resolved any discrepancies before the calls were finalized. Data generated on the ABI 3100 were normalized from runs of a set of samples on the ABI 3730 using the program Allelogram (Morin *et al.* 2009). The size of each allelic pair for each locus constituted the raw data for analyses.

Samples were genetically sexed using the zinc finger (ZFX and ZFY) genes. Prior to 2005, sex determinations were completed according to Fain and LeMay (1995). After 2005, a Real-Time PCR (Stratagene) assay was used as described in Morin et al. (Morin *et al.* 2005).

Data QA/QC

We adhered to the nuclear data quality assurance and quality control (QA/QC) protocol described by Morin et al. (2010). Samples that were missing data for >25% of the markers, were homozygous at nine or more loci or could not be consistently replicated, were deemed to be of poor quality and removed from the data set. Loci were tested for evidence of allelic dropout and null alleles using the program MICROCHECKER (version 2.2.3)(Van Oosterhout *et al.* 2004).

Deviation from Hardy-Weinberg equilibrium (HWE) was assessed for each microsatellite locus using GENEPOP version 4 (Rousset 2008). Both exact tests of HWE (Guo and Thompson 1992) and tests for heterozygote deficiency (Raymond and Rousset 1995) were conducted. The same software was used to evaluate linkage disequilibrium for each pair of loci using Fisher's method and the Markov chain method. All HWE and linkage disequilibrium tests were conducted using program defaults for the Markov chain parameters (1,000 dememorization steps, 100 batches, 1,000 iterations per batch). Fisher's method (Fisher 1935) was used to combine *P*-values across strata to calculate a global *P*-value for each locus (HWE) or locus pair (linkage disequilibrium). The jackknife procedure described in (Morin *et al.* 2010) was used to identify samples that were highly influential in deviations from HWE. Genotypes that had log-odds larger than two were removed from the data set.

We used the program Cervus 3.0.3 (Kalinowski *et al.* 2007) to examine our ability to discriminate unique individuals using our microsatellite data set. We calculated both the probability that two randomly chosen individuals would possess the same multi-locus genotype (PI) and the probability that full-siblings would share the same genotype (PIsibs). Pairs of samples that matched in sex, mtDNA haplotype, and microsatellite genotype were considered duplicate samples from the same individual and only one sample was retained in the dataset. The program DROPOUT (McKelvey and Schwartz 2005) was used to identify additional pairs of samples whose genotypes differed at four or fewer loci and therefore could represent duplicate samples with genotyping errors.

All samples with unique haplotypes (i.e., not present in any other sample) were re-sequenced two or more times to confirm the sequence. We reviewed the haplotype data published by Chivers et al. (2007) to ensure data quality. In cases where the sequences published by Chivers et al. were uncertain, we re-sequenced samples.

Genetic diversity

We used ARLEQUIN, version 3.5.1.2 (Excoffier *et al.* 2005) to calculate the haplotypic diversity (*h*) and nucleotide diversity (π) in the mtDNA dataset. We also used ARLEQUIN to calculate Tajima's D (Tajima 1989) and Fu's F_s (Fu 1997) in order to look for evidence of population expansion or bottlenecks. We used the program FSTAT (Goudet 2001) to calculate allelic richness (based on a minimum sample size of 20), number of alleles per locus and observed heterozygosity within the nucDNA dataset.

Population differentiation

We used *F*-statistics and tests for differences in alleles frequencies to examine population structure at two different scales within the Hawaiian Archipelago. We first stratified our samples according to whether they were collected in the MHI or the NWHI. We conducted these tests both with and without the NWHI samples collected on Oct. 7th because it is unclear whether these individuals are part of the same population as the other NWHI individuals in our sample set. Second, we examined genetic structuring within the MHI by stratifying samples according to the three large social clusters identified by Baird et al. (2011a). Baird et al.'s analyses also identified four small (between 2 and 16 individuals) peripheral social clusters, at least some of which are likely artifacts of the small number of observations for the animals contained in those clusters (Baird *et al.* 2011a). We therefore treated the social cluster affiliation of samples from those four clusters as unknown.

For each stratification, we used the χ^2 permutation test implemented in the R package eiaGenetics (available upon request) to test for differences in haplotype frequencies within the mtDNA data and allele frequencies in the nucDNA data. We used three *F*-statistics to estimate genetic differentiation between populations. For both data sets, we calculate Weir and Cockerham's (1984) θ, which we henceforth refer to as F_{ST} following standard convention. For the mtDNA, we also calculated Φ_{ST} (Excoffier *et al.* 1992), which takes into account the evolutionary distances between haplotypes. For the nuclear data set, we calculated F'_{ST} (=θ/θ_{max})(Hedrick 2005, Meirmans 2006). F'_{ST} corrects for within-population diversity, making it more appropriate for making demographic inferences than θ (Meirmans and Hedrick 2011). All *F*-statistics were calculated using the R package eiaGenetics.

We tested for evidence of sex-biased dispersal in the microsatellite data set using the biased dispersal test of FSTAT (Goudet 2001, Goudet *et al.* 2002). We examined differences between males and females with respect to mean and variance of assignment indices, F_{IS}, F_{ST}, relatedness, and within-group gene diversity (H_S) and assessed significance through 1,000 permutations. This tests looks for differences between the sexes in the number of immigrants present in the sample. It cannot detect male-mediated gene flow, i.e., gene flow due to inter-group mating without dispersal.

We looked for first generation migrants between our MHI and NWHI strata using the program GeneClass2 (Piry *et al.* 2004). We used the assignment criterion of Paetkau et al. (1995) to calculate the likelihood of each sample having originated in each population and assessed the significance of those likelihoods using Paetkau et al.'s (2004) re-sampling method. We used L_home as the likelihood value, as it is possible that some individuals could have originated in unsampled source populations. For all analyses, we set the default frequency for missing alleles at 0.01, performed 1,000 re-sampling events, and set the type I error rate to 0.01 as recommended by Piry et al. (2004).

Parentage analyses

We used Cervus 3.0.3 (Kalinowski *et al.* 2007) to identify putative parent-offspring pairs in our dataset. Cervus uses a maximum likelihood approach to identify putative parent-offspring pairs. It uses a simulation to determine critical values for the test statistic in order to ensure that all parent-offspring pairs it identifies meet a user-specific confidence level. We set the confidence level in our simulations at 95%. We specified the proportion of loci typed as 0.966 to match our actual dataset, and set the genotyping error rate at 0.01. We set the proportion of candidate parents sampled at 0.4, but examined sensitivity of the results to this parameter by re-running the analysis at 0.1. We ran paternity and maternity analyses separately. When attempting to identify parents for individuals sampled from the MHI, we calculated the population allele frequencies based only on MHI samples, and vice versa when assigning parents to NWHI individuals. In each case, individuals from both populations were included in the list of candidate parents.

In most cases Cervus was unable to resolve which member of a parent-offspring pair is the parent and which is the offspring. We therefore attempted to determine relative ages of individuals based on photographic data and sighting dates. Using the long-term photo-identification catalog (Baird *et al.* 2008), individuals were classified into stages (adults, sub-adults, or juveniles) based on markings on the dorsal fin, relative size in photographs, the number of years that have passed since they were first identified, and whether they have been observed with a small calf in close proximity. Mother/calf relations were only assigned when photos clearly showed a calf in infant position relative to the identified mother during surfacing. Individuals are given a designator in the catalog when they have sufficient markings to be identifiable from photographs, which typically requires acquisition of body scars or notches on the dorsal fin. Dorsal fin notch acquisitions or changes for this population have been estimated to occur on average every 6.9 to 8.8 years (Baird *et al.* 2008), thus individuals with notches are typically a year or more of age when they are first cataloged. An individual was deemed an adult once eight years had passed since it was first identified (either genetically or photographically). If eight years had not passed since it was first identified, all photographs of the individual when other individuals were visible in the photo were assessed to determine relative size (e.g., whether the

individual was approximately the same size as other known adults or smaller indicating it may be a sub-adult or juvenile). Given the slow acquisition of notches, the number of notches on the dorsal fin were also taken into account, with individuals with few or no notches and small relative size categorized as juveniles.

Effective population sizes

We estimated the effective population size (N_e) of different strata with the program LDNe (Waples 2006, Waples and Do 2008), which uses estimates of linkage disequilibrium (LD) to infer N_e. To investigate how estimated N_e varies across different levels of population structure and social structure, we estimated N_e for five different strata: the entire Hawaiian Archipelago, the MHI, and the three social clusters identified by Baird et al. (2011a) within the MHI. We examined the effects of sample size on the estimates of N_e by subsampling from the original dataset. Within each stratum, we drew subsamples ranging in size from 10 to the actual number of samples in that stratum. We repeated the subsampling ten times for each subsample size and calculated the harmonic mean N_e from the ten replicate subsamples. MHI social cluster 2 was not included in the subsampling due to small sample size (n=11; Table 2).

Results

Data QA/QC

The probability of identity for our nucDNA dataset was 1.95×10^{-17} for unrelated individuals and 4.4×10^{-7} for full-siblings, indicating that the microsatellite loci were adequate for identifying unique individuals. After duplicate and poor-quality samples were excluded, there remained 112 samples (91 from the MHI and 21 from the NWHI) in the nucDNA dataset and 117 samples (96 from the MHI and 21 from the NWHI) in the mtDNA dataset.

Both the probability test and the test for heterozygote deficiency showed that marker D12t deviated significantly from HWE within the MHI stratum, but not in the NWHI stratum. No other loci exhibited significant departures from HWE, nor did the MHI depart significantly from HWE when we combined *p*-values across markers. When we stratified the data into MHI and NWHI and then used Fisher's method to combine p-values across strata, twelve pairs of loci were found to be in linkage disequilibrium, and the global *p*-value across locus pairs was statistically significant. This result is not surprising given the strong social structure within the MHI (see below). However, when we examined linkage disequilibrium separately in each of the three MHI social groups and the NWHI and then combined across strata, only four pairs of loci showed linkage disequilibrium and the global test across loci was non-significant. No loci showed evidence of null alleles or allelic dropout. No samples were identified as outliers in the HWE jackknife analysis.

Re-sequencing of samples revealed errors in the Chivers et al. (2007) sequences and resulted in the elimination of two of the haplotypes (haplotypes 3 and 4) identified by Chivers et al. from the Hawaiian Insular population. The numbers assigned to those haplotypes were not re-used.

Genetic diversity

We detected four haplotypes in the mtDNA data set (Table 1), three of which correspond to haplotypes 1, 2, and 5 identified by Chivers et al. (2007, 2010). The fourth

haplotype was a new haplotype that differed from haplotype 1 by a single transition. It was designated haplotype 31. Haplotype 1 was most common, with frequencies of 0.760 in the MHI and 0.955 in the NWHI. Haplotype 2 was restricted to the MHI, where it had a frequency of 0.229. Haplotype 2 was not detected in any individuals from social cluster 3 (Table 3).

Haplotypes 5 and 31 were each only detected in a single individual. The individual with haplotype 31 was sampled twice in the NWHI, once on Sept. 26 and again on Oct. 21. The individual with haplotype 5 was only encountered once, in 2005, off the coast of Hawai'i Island. It was photographed and entered into the Hawaiian Insular photo identification catalog, but has not been seen again. Haplotype 5 is divergent from the other three haplotypes in our data set and has also been detected in animals sampled off of northern Australia (Chivers *et al.* 2010).

Table 1. Haplotype frequencies in the strata.

		Haplotype			
Stratum	**n**	**1**	**2**	**5**	**31**
NWHI	21	20	0	0	1
MHI	96	73	22	1	0
Social cluster 1	30	17	13	0	0
Social cluster 2	12	6	5	1	0
Social cluster 3	23	23	0	0	0

Both haplotypic and nucleotide diversity were low in the mtDNA data set (Table 2). We detected greater diversity in the nucDNA data set, with heterozygosity around 0.74. The number of alleles per locus ranged from 5.75 to 7.63. Both Tajima's D and Fu's F_s for the mtDNA data set were non-significant (*p*-value > 0.05) in both the NWHI and MHI.

Table 2. Summary diversity statistics for the different strata: n = sample size, k = number of alleles/haplotypes per locus, h = haplotypic diversity, π = nucleotide diversity, H_o = observed heterozygosity, and A_r = allelic richness, based on a minimum sample size of 20. Due to its dependence on sample size, allelic richness was not calculated within the social clusters.

	mtDNA				nucDNA			
Stratum	**n**	**k**	**h**	**π**	**n**	**k**	**H_o**	**A_r**
NWHI	21	2	0.095	0.0001	21	6.88	0.735	6.814375
MHI	96	3	0.373	0.0009	91	7.63	0.745	6.432375
Social cluster 1	30	2	0.508	0.0011	30	6.50	0.732	
Social cluster 2	12	3	0.621	0.0018	11	5.75	0.743	
Social cluster 3	23	1	0	0	23	6.38	0.745	

Population structure

MHI vs. NWHI – We found significant genetic differentiation between the MHI and NWHI in both the nucDNA (F_{ST} = 0.031, F'_{ST} = 0.121, χ^2 *p*-value < 0.001) and mtDNA (F_{ST} = 0.101, ϕ_{ST} = 0.092, χ^2 *p*-value = 0.001) datasets. When we excluded samples from the encounter on Oct. 7 from the NWHI, differentiation was slightly lower, though still

significant (for nucDNA F_{ST} = 0.028, F'_{ST} = 0.110, χ^2 p-value < 0.001; for mtDNA F_{ST} = 0.089, ϕ_{ST} = 0.081, χ^2 p-value = 0.032). None of the tests for sex-biased dispersal between the MHI and NWHI were statistically significant.

Most samples assigned strongly to the population from which they were sampled. Only six samples from the MHI and four samples from the NWHI had assignments to their home populations of less than 90% (Table 3). One of the NWHI samples (lab ID 102221) with a home-assignment probability less than 90% was collected Oct. 7th, while the remaining three were collected during one of the encounters (Sept. 26th) that Baird et al. identified as involving island-associated animals Only one sample, which was collected in the MHI, could be excluded from its home population with a p-value < 0.01 (Table 3).

Table 3. Assignment probabilities and exclusion P-values for samples that had less than 90% assignment probability to their home population, as well as one sample that was excluded from its home population.

Lab ID	Population (social group)	Hap ID	Sex	Assignment Probability		Exclusion *p*-value	
				MHI	NWHI	MHI	NWHI
102434	NWHI	1	M	0.358	0.642	0.005	0.014
102435	NWHI	1	M	0.480	0.520	0.012	0.26
102433	NWHI	1	F	0.879	0.121	0.038	0.023
102221	NWHI	1	M	0.655	0.345	0.276	0.264
33891	MHI	1	M	0.290	0.971	0.065	0.382
98743	MHI (2)	1	F	0.283	0.717	0.308	0.424
49044	MHI	1	F	0.471	0.529	0.355	0.515
30072	MHI	1	F	0.623	0.372	0.052	0.079
102485	MHI (3)	1	M	0.658	0.342	0.116	0.130
33903	MHI (3)	1	F	0.700	0.300	0.308	0.315
91086	MHI	1	F	0.929	0.071	**0.001**	0.001

<u>MHI social clusters</u> – Both the nucDNA and mtDNA datasets showed that social cluster three differed significantly from social clusters one and two (Tables 4 and 5). Differences between social clusters one and two, however, were not statistically significant in either mtDNA or nucDNA. None of the tests for sex-biased dispersal between the social clusters were statistically significant.

Table 4. Pairwise comparisons between MHI social clusters using nucDNA data. Sample sizes are shown in parentheses. (A) χ^2 p-values. (B) F_{ST} below the diagonal, F'_{ST} above the diagonal

(A)

Social group	1	2	3
1 (30)	NA		
2 (11)	0.1718	NA	
3 (23)	**0.0010**	**0.0090**	

(B)

Social group	1	2	3
1 (30)	NA	0.0356	0.0413
2 (11)	0.0093	NA	0.0726
3 (23)	0.0108	0.0185	NA

Table 5. Pairwise comparisons between MHI social clusters using mtDNA data. Sample sizes are shown in parentheses. (A) χ^2 p-values. (B) F_{ST} below the diagonal, phiST above the diagonal

(A)

Social group	1	2	3
1 (30)	NA		
2 (12)	0.4028	NA	
3 (23)	**0.0003**	**0.0005**	

(B)

Social group	1	2	3
1 (30)	NA	-0.0326	0.3795
2 (12)	-0.04988	NA	0.4165
3 (23)	0.3795	0.4940	NA

Parentage analysis

Cervus identified 60 parent-offspring pairs that met the 95% confidence level (Table S1). Eight of the pairs involved individuals from the NWHI, while the remaining 52 involved individuals from the MHI. There were no pairs involving individuals from different strata (MHI and NWHI). In all female-female (FF) pairs, both individuals had the same mtDNA haplotype, as expected.

Within the MHI, there were 15 FF pairs, representing mothers/daughters, 11 male-male (MM) pairs, representing fathers/sons, and 26 female-male (FM) pairs, which could be either mothers/sons or fathers/daughters. For those pairs in which the social cluster membership of both individuals was known, we categorized the pairs according to whether

the two individuals were from the same social cluster or different social clusters (Figure 2). All of the FF pairs involved individuals that belonged to the same social cluster, whereas two out of five MM pairs involved individuals from different social clusters. Ten of thirteen (77%) FM pairs involved individuals from the same social cluster.
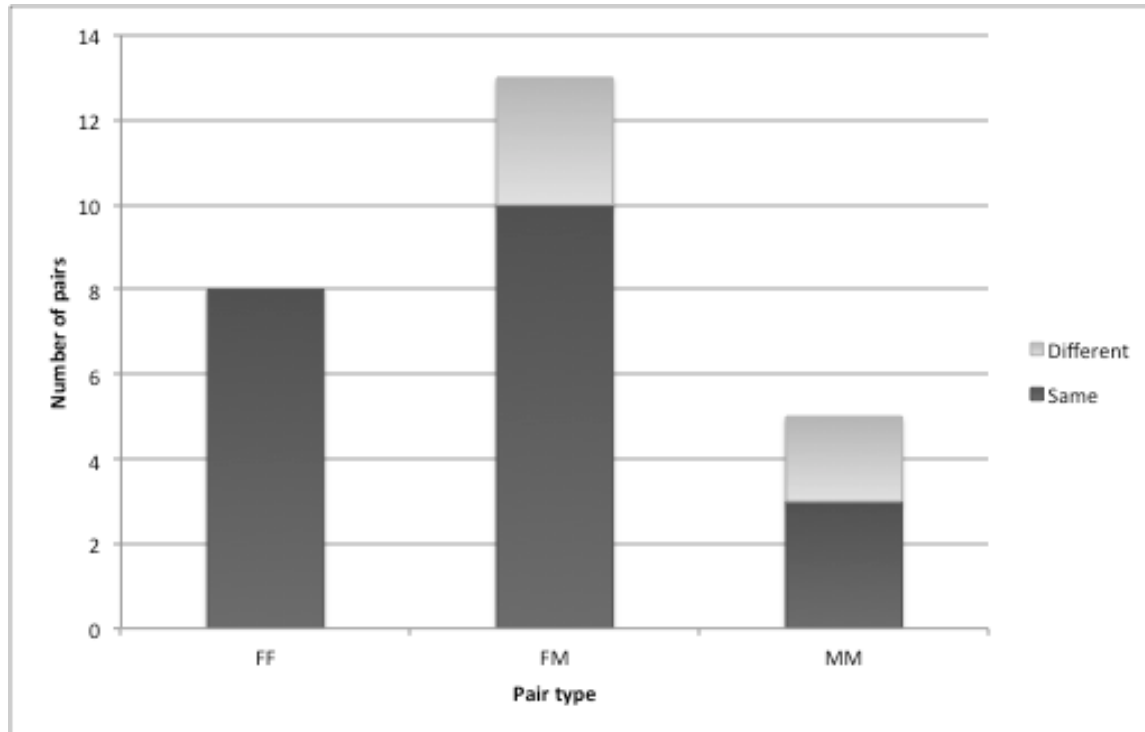

Figure 2. Number of parent-offspring pairs in which the two individuals come from the same or different social clusters. Only pairs in which the social cluster affiliation of both individuals is known are shown. Pairs are divided into female-female (FF) pairs, which represent mothers and daughters, male-male (MM) pairs, representing fathers and sons, and female-male (FM) pairs, which could be mothers and sons or fathers and daughters.

We used photographic data and sighting dates to assign individuals from the MHI to stage categories (Table S2). We were able to use these data to determine which individual was the parent and which was the offspring for eleven of the pairs identified by Cervus, including three FM pairs (Table S1). Two pairs resolved as father/daughter pairs, one of which (98743/102485) involved individuals from different social clusters, while the other (91277/23317) involved a daughter whose social cluster affiliation was unknown. One pair (91284/18938) resolved as a mother/son pair, but the social cluster affiliation of the mother was unknown.

One of the MM pairs identified by Cervus involved the only individual (individual 49052) in our data set that possessed haplotype 5. This individual, a male sighted with social cluster 2, was identified as the father or son of individual 98745, also a male from social cluster 2. Individual 49052 was identified as an adult in photographs taken in 2005, the only time the individual was sighted, while individual 98745 was identified as a probable sub-adult from photographs taken in 2010. Thus, it is likely that individual 49052 is the father of individual 98745.

Several of the MHI individuals that had less than 90% assignment probability to the MHI in the assignment test (Table 3) were identified by Cervus as parent-offspring pairs, namely 49044/30072, 30072/98743, and 98743/102485 (Table S1). We were able to use photographic data to identify individual 98743 as the offspring in both pairs in which it was involved (Table S1), thus indicating that it is the daughter of 30072 and 102485. We were not able to determine the relative ages of 49044/30072 (Table S2).

Effective population size

Estimates of effective population size were low for all strata examined (Table 6). Subsampling indicated that effective population size estimates for the MHI and the whole Hawaiian Archipelago were relatively insensitive to sample sizes larger than 30-40, but that estimates for social clusters one and three varied with sample size up to and including the actual sample size, indicating they are liable to change as samples are added (Figure 3).

Table 6. Estimates of effective population size within different strata. 95% CIs were generated by jackknifing.

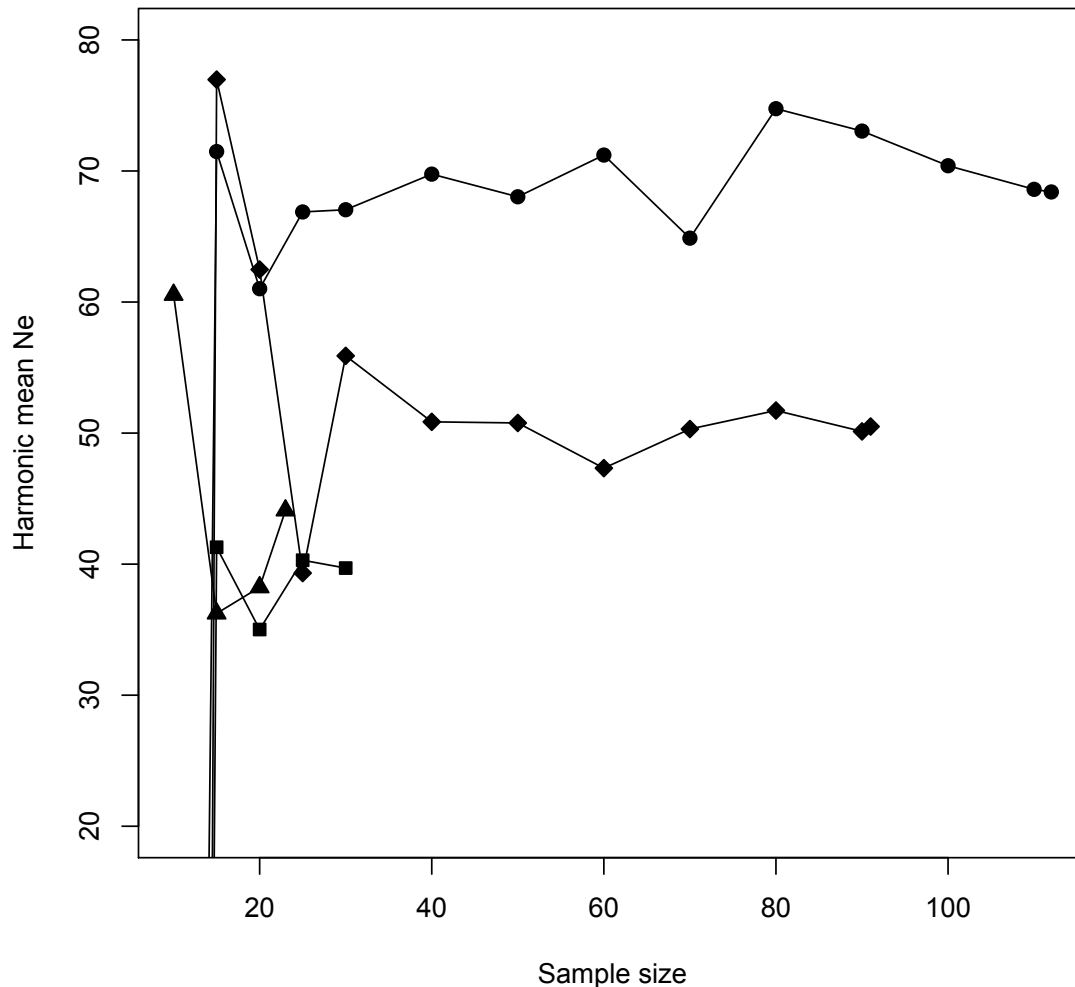| Stratum	n	Ne	CI
Hawaiian Archipelago	112	68.4	58.8-80.5
MHI - all samples	91	50.5	43.5-59.3
MHI - social cluster 1	30	39.7	29.5-57.7
MHI - social cluster 2	11	45.5	18.6-infinity
MHI - social cluster 3	23	44.1	28.3-87.1

Figure 3. Estimates of Ne for different strata as a function of sample size. Points represent the harmonic mean of estimates across 10 replicate subsamples. The last point in each data series indicates the point estimate of Ne obtained using all samples available for a given stratum. Circles show estimates for the entire Hawaiian archipelago, diamonds show the main Hawaiian Islands, squares show social cluster 1, and triangles show social cluster 3.

Discussion

Population differentiation across the Archipelago

Our results suggest that the island-associated animals sampled in the NWHI represent a demographically-independent population that is distinct from the Hawaiian Insular population. Both the mtDNA and nucDNA datasets revealed significant differentiation between the two populations. The mtDNA differentiation is driven by the fact that haplotype 2, which comprises 23% of the sample from the MHI, is completely absent from the NWHI. This marked difference in frequencies indicates very limited female dispersal between the populations.

The nucDNA differentiation between the MHI and NWHI (F_{ST} = 0.031) is comparable to or greater than that observed between other groups of Hawaiian cetaceans that are managed as separate stocks. Martien et al. (2011) reported nucDNA F_{ST} s ranging from 0.007 to 0.013 between populations of bottlenose dolphins (*Tursiops truncatus*) from the different Hawaiian island groups, while Andrews et al. (2010) reported nucDNA F_{ST} values between genetically distinct stocks of Hawaiian spinner dolphins (*Stenella longirostris*) ranging from 0.009 to 0.031. Furthermore, nucDNA differentiation between the MHI and NWHI was comparable to that observed between the MHI populations and samples from distant locales within the eastern North Pacific, including Mexico, Panama, and American Samoa (Chivers *et al.* 2010, Chivers *et al.* 2011).

The independence of the MHI and NWHI populations is further supported by the strong assignment of most individuals back to the population from which they were sampled. The only individual that could be excluded from the population in which it was sampled (MHI) did not assign strongly to the other population, indicating that it most likely is a migrant from a population that was not included in the assignment analysis.

Though both the mtDNA and nucDNA data support the conclusion that the NWHI and MHI strata represent distinct populations of false killer whales, our sample distribution within the NWHI is too limited to allow us to evaluate population structure within the NWHI. Three groups included in our NWHI stratum are linked by photo identification and satellite tracking data (Baird *et al.* 2011b), and therefore likely involve individuals that all belong to the same population. However, we do not have sufficient data to allow us to determine whether the fourth NWHI group included in our study belongs to this population or another, undocumented population in the vicinity. Furthermore, we do not know whether island-associate false killer whales are present in the western portion of the NWHI or, if so, how they relate to the animals included in this study.

Social structure and mechanism of gene flow

Our parentage analyses indicate that social structure within the MHI population is based on philopatry to natal social cluster. All female-female (FF) pairs involved individuals from the same social cluster, demonstrating that female offspring remain in the same social cluster as their mothers. The only female-male (FM) pair that we were able to resolve as mother/son involved a mother whose social cluster affiliation was unknown. However, of the thirteen female-male (FM) pairs in which the social cluster of both individuals was known, only three involved individuals from different social clusters, and one of those was resolved as a father/daughter pair. Thus, it appears that neither males nor females tend to disperse from their natal social clusters in substantial numbers. This conclusion is supported by the non-significance of the tests for sex-biased dispersal, though the statistical power of that test is known to be low (Goudet *et al.* 2002).

We identified six father/offspring pairs (five MM pairs and one FM pair that resolved as father/daughter) in which the social cluster affiliation of both individuals was known. Three of these pairs involved individuals from the same social cluster, indicating that mating occurs both within and between social clusters. This pattern is similar to that seen in southern resident killer whales (*Orcinus orca*; Ford *et al.* 2011), but contrasts with mating patterns inferred for northern and Alaskan resident killer whale populations (Barrett-Lennard 2000, Pilot *et al.* 2010). In the latter case, mating occurred almost exclusively between individuals from different social groups. In species with strong fidelity

to natal social groups, breeding within social groups could result in inbreeding depression due to the high probability of breeding between close relatives. Inbreeding avoidance was proposed as the likely explanation for the mating patterns observed in northern and Alaskan resident killer whales (Barrett-Lennard 2000, Pilot *et al.* 2010). Ford et al. {, 2011 #377} suggest that restricted mate choice due to the small size of the southern resident killer whale population may have contributed to the breakdown of this inbreeding avoidance mechanism in that population

Effective population size

We estimate that the N_e of the MHI insular false killer whale population is about 50 animals. This population is probably naturally small with strong social structure that limits genetic diversity. Nonetheless, such a low estimate of N_e is cause for concern, as domestic animal studies show that lethal or semi-lethal genetic traits begin to be displayed when N_e declines to about 50 individuals (Franklin 1980). The estimate of N_e produced by LDNe is influenced by the effective size of the population in the generations immediately prior to the collection of samples. Although no data are available for calculating trends in abundance, observational data suggest abundance of the MHI insular population may have declined precipitously over the last two decades (Baird 2009, Reeves *et al.* 2009, Oleson *et al.* 2010). If such a decline has occurred, then N_e is likely to decline in coming decades as the population comes into equilibrium at a new, lower population size.

There are several potential sources of uncertainty in our estimate of N_e. Subsampling showed that, though our estimates of N_e in the MHI population and the Archipelago as a whole are robust to the addition of more samples, sample sizes from the individual MHI social clusters are too small to allow reliable estimation of N_e. Thus, additional samples may result in changes to the estimates for these strata. Furthermore, LDNe is known to have a slight (<5%) negative bias (Waples 2006), and was developed and tested under the assumption of a closed population with non-overlapping generations. The bias, if any, introduced by overlapping generations has not been well studied, although Waples (2006) notes that analyses of populations with overlapping generations will estimate the effective number of breeders that produced the sample, which is related to N_e.

If a population is not completely closed, but rather receives immigrants from other populations, the estimate of N_e will be positively biased due to a Wahlund effect created by the presence of first-generation immigrants in the population. The amount of linkage disequilibrium introduced by this effect is small (Waples and Smouse 1990) and therefore unlikely to significantly impact estimates of N_e. We examine the impact of violating the assumption of a closed population by estimating N_e at three different scales – social groups within the MHI, the entire MHI population, and the entire Hawaiian Archipelago. The 95% confidence intervals for most of the estimates were too broad to allow us to draw robust comparisons across scales. Nonetheless, the general trend was for estimates of N_e to increase with increasing scale, which is consistent with the amount of genetic structuring within strata increasing as the scale progresses from social groups to a metapopulation.

Conclusions and management implications

Our genetic results corroborate Baird et al.'s conclusion based on photo identification and tagging data that there is a previously undocumented island-associated population of false killer whales in the eastern portion of the NWHI. This population is

genetically distinct at both mtDNA and nucDNA markers from the Hawaiian Insular population, represented here by our MHI stratum. The degree of differentiation between these two populations is sufficient to warrant management as separate stocks.

Parentage analyses for the Hawaiian Insular population indicate that both males and females exhibit philopatry to natal social clusters, with mating occurring both within and between social clusters. This form of social structure has the potential for increasing the risk of deleterious genetic traits due to inbreeding. This possibility is particularly alarming given the small estimated effective population size of this population (N_e=50.5) and the suggestion that its abundance may have declined dramatically in the last twenty years. If such a decline has occurred that recently, our estimates of N_e still reflect the pre-decline condition of the population.

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Supplemental Material

Table S1. Parent/offspring pairs identified with high confidence by Cervus. For those pairs in which the relative ages of the individuals could be determined from photographic and sighting data, the ID of the individual identified as the parent is in bold. The first six columns provide the sample identification numbers, sex, and haplotype for the two individuals in the pair. The columns labeled 'Soc1' and 'Soc2' give the social group affiliation for MHI individuals. A blank indicates that the social cluster affiliation is unknown, while 'NWHI' indicates the individual is from the NWHI population. 'Loci' indicates the number of microsatellite loci at which both individuals were scored and could therefore be compared and 'Mis-match' indicates the number of loci at which the two individuals do not share at least one allele. Mismatches can occur due to mutation, genotyping error, or incorrect assignment of parentage.

ID1	ID2	Sex1	Sex2	Hap1	Hap2	Soc1	Soc2	Loci	Mis-match
18945	75677	F	F	2	2	1	1	13	1
18954	33907	M	M	1	1		3	16	0
18955	91083	F	M	1	1			16	0
23316	23321	M	F	1	1	1	1	16	0
23316	98746	M	M	1	2	1	2	16	0
23317	91277	M	F	1	1	1		13	0
23317	92256	M	M	1	1	1	1	15	0
23318	30078	F	M	2	2	1		15	0
23318	75666	F	F	2	2	1		16	0
23320	98738	F	M	2	2	1	1	16	0
27453	27454	F	F	1	1	3		16	0
30072	98743	F	F	1	1		2	16	0
30073	23321	M	F	1	1	2	1	16	1
30077	49054	M	F	1	1			15	1
30077	102483	M	M	1	1		3	14	0
30081	49049	F	M	1	1			13	0
33886	45932	M	M	1	1	3	3	16	0
33886	75666	M	F	1	2	3		16	1
33887	123188	F	F	1	1	3	3	12	2
33888	30078	F	M	1	2	3		14	0
33890	30078	F	M	1	2	3		15	0
33890	123188	F	F	1	1	3	3	15	0
33892	33909	F	M	1	1	3	3	14	0
33895	45932	F	M	1	1	3	3	16	0
33902	33908	F	M	1	1	3	3	16	2
33902	45928	F	F	1	1	3	3	15	0
33903	33904	F	M	1	1	3		14	0
33907	98736	M	F	1	2	3	1	16	0

45925	75666	M	F	2	2	2		14	0
49044	30072	F	F	1	1			13	1
49046	49051	F	F	2	2	2	2	15	1
49047	30078	F	M	2	2			14	0
49047	49051	F	F	2	2		2	14	1
49051	98746	F	M	2	2	2	2	15	0
49052	98745	M	M	5	2	2	2	13	0
71016	71017	M	F	2	2	1	1	16	0
75661	33886	M	M	1	1	1	3	16	1
75663	30078	M	M	2	2	1		15	1
75666	75677	F	F	2	2		1	16	0
75676	75679	F	F	1	1	1	1	16	0
75678	75679	F	F	1	1	1	1	16	0
75679	92256	F	M	1	1	1	1	16	0
91276	91277	F	F	1	1			14	0
91284	**18938**	M	F	1	1			14	1
91284	**23317**	M	M	1	1		1	14	0
98732	98737	F	F	2	2	1	1	16	0
98740	30078	F	M	1	2	1		15	0
98743	**102485**	F	M	1	1	2	3	16	0
98744	**30078**	M	M	1	2	2		15	0
102500	23318	M	F	1	2	1	1	16	1
102500	30078	M	M	1	2	1		15	0
123188	33907	F	M	1	1	3	3	15	1
102218	102219	F	F	1	1	NWHI	NWHI	16	0
102222	102233	F	F	1	1	NWHI	NWHI	16	0
102223	102222	M	F	1	1	NWHI	NWHI	16	0
102224	102222	M	F	1	1	NWHI	NWHI	16	0
102226	102218	F	F	1	1	NWHI	NWHI	16	2
102230	102233	F	F	1	1	NWHI	NWHI	16	0
102436	102438	F	F	1	1	NWHI	NWHI	16	0
102437	102438	M	F	1	1	NWHI	NWHI	16	0

Table S2. Stage categories of individuals based on photo-identification data. Categories are A=adult, SA=sub-adult and J=juvenile. Stages prefaced with P are probable categories. Stage categories given are for the most recent year seen, unless a year is noted in parentheses. The column labeled 'Basis' indicates the data on which the age determination is based. IC=in catalog more than eight years, M=markings, RS=Relative size in photographs with other individuals present, and W/C means with calf (year seen with calf in parentheses). Distinctiveness of the individual was rated as 4=very distinctive, 3=distinctive, 2=slightly distinctive, 1=not distinctive.

ID	First seen	Last seen	# years seen	# times seen	Distinctiveness	Stage category	Basis
18945	1988	2010	9	21	4	A	IC
18946	2000	2010	7	10	4	A	IC
18954	2000	2000	1	1	2	P A	M
18955	2000	2008	2	3	3	A	IC
23316	2001	2010	7	16	4	A	M / IC
23317	2001	2010	6	8	4	A	IC
23318	2000	2010	5	13	2	A	IC, W/C (2008)
23319	1990	2010	10	15	4	A	IC
23320	1999	2008	6	10	4	A	IC
23321	2001	2010	6	15	4	A	M, W/C (2006)
27453	2002	2010	3	3	1	P A	RS, W/C
30073	2002	2004	2	2	4	A	M
33885	2003	2010	4	7	4	A	M/RS
33886	2003	2003	1	1	4	A	M
33887	2003	2010	5	7	4	A	RS, M
33888	2003	2010	4	6	3	P SA	RS, M
33890	2002	2003	2	2	1	P SA	M
33892	2002	2003	2	2	4	A	M
33895	2003	2010	5	7	3	A	W/C (2009)
33898	2003	2010	5	8	3	P A	RS, M
33899	2003	2007	3	4	3	A	W/C (2007)
33902	2003	2008	3	4	3	P SA	M
33903	2003	2010	5	6	4	A	M/RS
33905	2003	2010	4	4	3	P A	RS
33906	2003	2009	4	5	3	A	M
33907	2003	2004	2	2	3	A	M
33908	2003	2010	5	9	4	A	RS, M
33909	2003	2004	2	2	4	P A	M
41286	2001	2010	7	10	4	A	IC
45925	1986	2010	6	6	4	A	IC
45928	2003	2010	4	5	4	A	RS, M
45932	2003	2010	4	5	4	A	M
49043	2005	2010	2	2	4	P A	M
49046	2005	2010	2	2	4	A	RS, M
49048	2002	2009	3	3	3	P J	M
49050	2004	2008	3	3	4	A	M

49051	2005	2010	2	2	3	A	M
49052	2005	2005	1	1	3	A	M
71016	2005	2010	5	14	3	P A	RS
71017	2000	2010	8	17	4	A	IC
73895	2008	2008	1	1	3	P A	M
75660	2003	2010	6	7	3	P A	RS, M
75661	2008	2008	1	5	3	P A	M
75662	1990	2010	9	13	4	A	IC
75663	2000	2010	7	13	4	A	IC
75665	1991	2010	12	16	4	A	IC
75676	2005	2010	6	19	3	P A	RS
75677	2008	2010	3	12	1	J (2008)	RS
75678	2007	2010	4	9	2	P J (2007)	M
75679	2005	2010	6	16	3	P J	M
91081	2006	2009	2	4	2	P SA	RS, M
91082	2009	2009	1	3	3	A	W/C
91083	1999	2009	3	3	4	A	IC, M
91084	2009	2009	1	1	4	A	M
91085	2000	2009	2	3	3	A	IC
91086	2009	2009	1	1	1	J	RS
91274	2004	2009	2	3	4	A	M
91275	2009	2009	1	1	2	J	RS, M
91276	2009	2009	1	1	3	A	W/C
91277	2009	2009	1	1	1	J	RS
91278	2009	2009	1	1	2	P A	M
91284	2009	2009	1	3	1	J	RS
91285	2008	2011	4	15	1	J (2008)	M/RS
92254	2009	2010	2	6	1	P J	M
92256	2007	2010	4	14	3	P J (2007)	M
98732	2004	2010	7	17	3	P A	RS
98734	2005	2010	3	6	4	A	M
98735	1995	2010	10	15	4	A	IC
98736	2004	2010	7	21	4	A	M/RS
98737	2008	2011	5	14	1	P J (2008)	M
98738	1999	2010	10	18	4	A	IC
98740	2000	2010	6	9	3	A	M, IC
98743	2010	2010	1	1	2	P J	RS, M
98744	2010	2010	1	1	3	P SA	M
98745	2009	2009	1	1	2	P SA	M
98746	2005	2010	4	4	3	P A	M
102483	2008	2010	2	4	4	J (2008)	RS
102485	2004	2010	3	5	4	A	M
102500	2005	2010	6	10	3	A	RS
123188	2004	2010	3	4	3	P A	M