



Redrawing the map: mtDNA provides new insight into the distribution and diversity of short-finned pilot whales in the Pacific Ocean

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ABSTRACT

Correlations between morphological and genetic data provide evidence to delineate species or evolutionarily significant units, which then become the units to conserve in management plans. Here, we examine the distribution and genetic differentiation of two morphotypes of short-finned pilot whale (*Globicephala macrorhynchus*) in the Pacific Ocean. Mitochondrial control region sequences from 333 samples were combined with 152 previously published sequences to describe genetic variability globally and population structure in the Pacific. Although genetic variability is low, we found strong differentiation at both broad and local levels across the Pacific. Based on genetics, two types are distributed throughout the Pacific, one predominantly in the eastern Pacific and the other in the western and central Pacific. In the eastern Pacific Ocean, no correlation was found between distribution and sea surface temperature. The two types have broad latitudinal ranges, suggesting their distributions are likely driven by more complex factors, such as prey distribution, rather than sea surface temperature.

Key words: short-finned pilot whale, *Globicephala macrorhynchus*, phylogeography, distribution, genetics, southern form, northern form.

Comparative morphology has traditionally been the primary method for the identification of species. In recent years, however, genetic data have played an increasingly important role in taxonomic studies. Though genetic and morphological data are often complementary, the relationship between genotype and phenotype can be complex. Morphological traits, for example, have been found to be ecologically plastic in many cases (*e.g.*, Inoue *et al.* 2013, Scharnweber *et al.* 2013), and convergent evolution can confound the relationship between morphology and genetic structure. For example, dolphin species in four different families have evolved similar phenotypes to live in freshwater, and echolocation has developed twice in mammals through similar

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evolutionary pathways (Parker *et al.* 2013). For these and a variety of other reasons, some geneticists caution against using differences in morphology alone to classify populations (Allendorf and Luikart 2011).

However, in many cases genetic and morphological patterns are correlated (*e.g.*, Punnett 1904), making it possible for morphological variability to be used as a proxy for genetic variability and *vice versa*. These correlations can be useful in identifying barriers to dispersal or gene flow in elusive species such as cetaceans, and in some cases can support the taxonomic delineation of undiscovered or cryptic species, subspecies, or evolutionarily significant units (Taylor 2005).

The short-finned pilot whale (*Globicephala macrorhynchus*) inhabits both coastal and pelagic waters in tropical and subtropical regions globally. Two morphotypes have been identified in the Pacific Ocean (Yamase 1760, Kasuya *et al.* 1988). These morphotypes have been referred to as the northern and southern types based on their distribution off Japan, where they were first described (Kasuya *et al.* 1988). However, because their distribution outside of Japan is poorly known, we will refer to them by names originally ascribed to them by Yamase (1760): the northern type is called *Shiho goto* (goto meaning short-finned pilot whale) and the southern type is called *Naisa goto*. They differ in body size, melon shape, color pattern, and number of teeth. The Shiho type is larger and has a round melon and light saddle patch with a distinct posterior boundary, while the Naisa type is smaller with a square melon and a darker, indistinct saddle patch (Yonekura *et al.* 1980, Kasuya *et al.* 1988, Miyazaki and Amano 1994). Gray (1846) designated these forms as different species. The Naisa type was called *G. sieboldii* (Gray 1846), and the Shiho type was named *G. sibo* (Gray, 1846). A third species, *G. scammonii* (Cope 1869) with a skull morphology similar to that of the Shiho type of northern Japan, was described from a pilot whale collected off Baja California, Mexico. These three nominal species were all subsequently considered as junior synonyms of *G. macrorhynchus* (van Bree 1971).

Off the coast of Japan, the distributions and breeding phenology of the Shiho and Naisa types are well documented and largely nonoverlapping (Kasuya and Marsh 1984, Kasuya *et al.* 1988, Yonekura *et al.* 1980). Several studies indicate that they are separated by the Kuroshio-Oyashio Extension (KOE) Current, which influences the distribution of several other species (Briggs 1974, Kasuya *et al.* 1988), with the Naisa type inhabiting the warm-temperate waters of the Kuroshio Current while the Shiho type inhabits areas with cold-temperate waters of the Oyashio Current (Kasuya *et al.* 1988). The closely related long-finned pilot whale (*Globicephala melas*) has been shown to exhibit similar temperature-driven population structure in the North Atlantic (Fullard *et al.* 2000). However, some evidence suggests that the relationship between sea surface temperature (SST) and the geographic boundaries of these two types off Japan is weak, and is likely a proxy for other drivers (Kasuya *et al.* 1988). In addition to being geographically segregated off Japan, their breeding seasons do not overlap: the peak breeding season of the Shiho type animals (September) is later than that of the Naisa type animals (May) (Kasuya and Marsh 1984). These aspects of their ecology indicate that the animals are likely reproductively isolated, and it has been suggested that the two types may be distinct subspecies off the coast of Japan (Kasuya 1986, Kasuya *et al.* 1988, Wada 1988).

Previous studies suggest that the distributions of the Shiho and Naisa morphotypes extend beyond Japan. Naisa morphotypes have been found in Southeast Asia (Chen *et al.* 2014), Hawai'i (Polisini 1980; RWB, unpublished data) and the North Atlantic Ocean (RLB, unpublished data), and Shiho morphotypes have been found in the California Current and off Peru (Cope 1869, Polisini 1980, Reyes-Robles 2009).

These studies suggest a tropical form with Naisa type morphology and a cold-temperate form with Shiho type morphology (Polisini 1980, Kasuya *et al.* 1988). However, the full ranges of the morphotypes are not known due to a lack of morphological data in much of their geographic range, and published data to support this hypothesis are limited from many areas (*e.g.*, Eastern Tropical Pacific [ETP] and Hawai'i).

Kage (1999) found that morphology was strongly correlated with genetics for the two morphotypes off the coast of Japan, although his sample size was small for the Shiho morphotype. Oremus *et al.* (2009) showed that all Naisa haplotypes were characterized by a diagnostic substitution, and therefore suggested that the Naisa type may be a distinct subspecies. While these studies provide support for the delineation of Shiho and Naisa type short-finned pilot whales off the coast of Japan, they did not have data to describe the distribution of the two types of short-finned pilot whales in other parts of the North Pacific. Genetic analysis of short-finned pilot whales from throughout the Pacific Ocean could improve our understanding of the distribution of these two types, especially in data-deficient areas such as Hawai'i and the eastern Pacific Ocean (ETP and California Current).

In this study, we examine the population structure of short-finned pilot whales in the Pacific Ocean using new genetic samples, primarily available from the eastern Pacific Ocean and Hawai'i, as well as SE Asia and the South Pacific, Indian and western North Atlantic Oceans. We combine these new genetic samples with existing sequence data (Oremus *et al.* 2009) to examine global patterns of genetic diversity and distribution. We further investigate whether any patterns of genetic differentiation corroborate the current hypothesis of a SST-based distribution of the Shiho and Naisa types (*e.g.*, Kasuya *et al.* 1988), with special consideration for areas where morphological data are limited, such as the ETP and Hawai'i. Finally, we use our combined data set to improve our understanding of interbasin divergence in addition to divergence within the Pacific Ocean. Geographically separated distributions could support the classification of these two types as distinct species or subspecies.

METHODS

Sample Collection

DNA sequences from *G. macrorhynchus* were generated from samples in the Southwest Fisheries Science Center (SWFSC) Marine Mammal and Turtle Molecular Research Sample Collection or obtained from GenBank (Benson *et al.* 2006). The samples archived at SWFSC ($n = 333$, Fig. 1) were collected between 1984 and 2011, primarily from biopsies taken during SWFSC research cruises along the west coast of the United States, in the ETP, the Northwest Hawaiian Islands, and Guam. Seventy-two samples were collected by Cascadia Research Collective from the main Hawaiian Islands as part of a long-term photo-identification study (Mahaffy *et al.* 2015, Baird *et al.* 2013). Additional samples were collected from strandings in New Zealand, Cambodia, Hawai'i, and the Pacific, Atlantic, and Gulf of Mexico coasts of the United States, and from fisheries bycatch off the coast of California and in the Philippines. Field sampling protocols included identifying individuals visually or with photographs to minimize resampling of individuals. Samples were stored at -80°C , or fixed in either a salt-saturated 20% DMSO solution or 100% ethanol and permanently archived in a -20°C freezer.

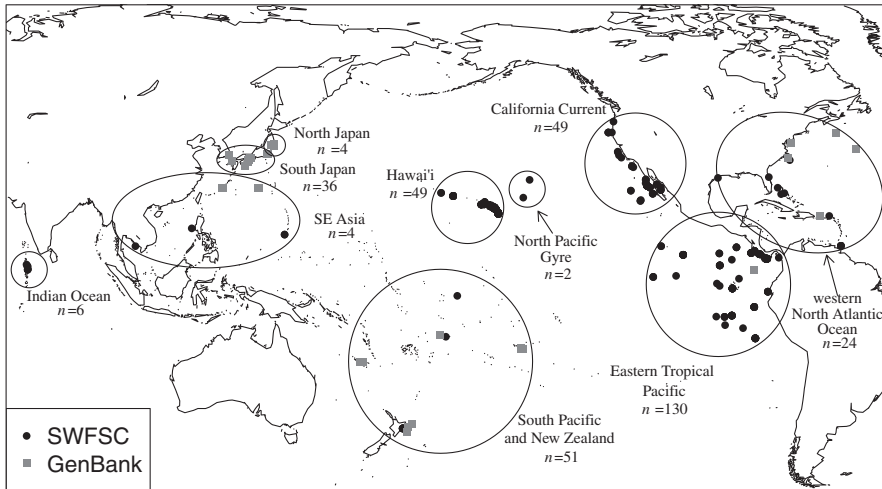


Figure 1. Sampling locations for all samples of short-finned pilot whale used in the present study. Circles represent the location of samples from the SWFSC Marine Mammal and Turtle Molecular Research Sample Collection. Squares indicate the approximate location of sequences taken from GenBank, based on locations published in Oremus *et al.* (2009). Ellipses delineate the strata used for tests of population differentiation; the break between the ETP and California Current represents a natural break in the known distribution of short-finned pilot whales. The two samples collected northeast of the Hawaiian Archipelago (“North Pacific Gyre”), and the market samples from Japan, were excluded from all statistical analyses except for the Median Joining Network.

Additionally, 15 tooth samples were obtained from skulls housed at the Los Angeles County Museum, and one from the American Museum of Natural History, in order to test the connection between morphology and haplotype in short-finned pilot whales outside of Japan.

DNA Sequencing

DNA was extracted from skin and muscle samples using a sodium chloride precipitation protocol (Miller *et al.* 1988), Qiagen DNeasy Blood and Tissue Kit (#69506, Qiagen, Germantown, MD) or a phenol-chloroform protocol (Sambrook *et al.* 1989). The hypervariable mtDNA control region was amplified and sequenced in two parts of approximately 420 bp and 560 bp, with approximately 20 bp of overlap between the two sequences. Primers, PCR, and sequencing methods have been described previously by Martien *et al.* (2014). The resulting combined sequence was 962 base pairs long, and was assembled using SEQED, version 1.0.3 (ABI), Sequencher software (versions 4.1 and 4.8; Gene Codes, Ann Arbor, MI) or Geneious (version 6.1.5, Biomatters Ltd, Auckland, New Zealand). New sequences were accessioned into NCBI GenBank with accession numbers KM624040-KM624062.

Short-finned pilot whale sequences from the 5' end of the control region that were already published in GenBank ($n = 152$, Fig. 1) were included to better represent global pilot whale haplotypic diversity. The majority of these sequences were from a study of the phylogeography of pilot whales in the western Pacific Ocean (Oremus

et al. 2009; FJ513328.1-FJ513341.1), with additional sequences from Vilstrup *et al.* (2011; JF339974-JF339976), Siemann (1994; U20921-U20923), Baker (direct submission, DQ145030-DQ145033), and Caballero *et al.* (2008; EU121124). The sequences acquired from GenBank were 345 bp in length; for the analyses that included these samples, all SWFSC sequences were truncated to match.

Tooth samples were extracted and sequenced according to the methods outlined in Morin *et al.* (2006). Two primer sets were used to sequence a 300 bp segment of the 5' mitochondrial control region: TRO (5'-CCTCCCTAAGACTCAAGGAAG-3', developed at SWFSC) and A3 (5'-AATACGRGCTTTAACT-3', Rosel *et al.* 1994) formed the first set; the second set consisted of D (5'-CCTGAAGTAAGAACA-GATG-3', Rosel *et al.* 1994) and A3r (5'-GATAAGTTA AAGCTCGTATT-3', developed at SWFSC).

Data Analysis

New sequences were aligned using a MAFFT alignment with default parameters (scoring matrix: 200PAM/k = 2, gap open penalty: 1.53, offset value: 0.123) in the Geneious software package (Katoh and Kuma 2002). Once the alignment was completed, sequences were reexamined. Any haplotypes represented by only a single sequence or haplotypes with a single base-pair difference from the most similar haplotype were reviewed for accuracy. Unique haplotypes were repeat sequenced in order to ensure the accuracy of the sequence. Once the newly generated sequences were verified, they were aligned with previously published GenBank sequences using the MAFFT alignment described above.

Samples were stratified according to their sampling location, resulting in nine broad geographic strata that were used for analysis of genetic differentiation (Fig. 1). These strata were chosen, wherever possible, based on natural breaks in the distribution of short-finned pilot whales (*e.g.*, the break in distribution between the ETP and California Current (Hamilton *et al.* 2009), and the break between north and south Japan); where this information was not available, strata were defined based on breaks in the sampling distribution. Two samples collected between Hawai'i and the North American coast ("North Pacific Gyre," Fig. 1) were not included in any statistical analyses due to their geographic isolation from all other samples. Sequences from Japanese market samples (Oremus *et al.* 2009) were also excluded from all statistical analyses because neither the geographic origin nor morphology of the animals from which they came was known. Therefore, the two North Pacific Gyre samples and all Japanese market samples were used only in the geographic analysis and median-joining network. Because short-finned pilot whales are known to exhibit social structure and may associate closely with relatives, there exists the potential for nonindependence in the full data set. Therefore, the data set was adjusted to include one sample of each haplotype from each encounter, in order to control for nonindependence, for the diversity and pairwise distance calculations.

Haplotype (h) and nucleotide (π) diversity, theta (θ_H , a measure of genetic diversity based on population size and mutation rate), and pairwise estimates of population differentiation (F_{ST} , Φ_{ST}) were calculated in Arlequin (Excoffier and Lischer 2010) for the adjusted data set using the short sequences (345 bp). The North Japan stratum was removed from pairwise analyses due to small sample size in the adjusted data set ($n = 1$). For F_{ST} , we calculated the genetic distance between haplotypes using the Tamura-Nei model with invariant sites, without a gamma correction (Tamura and Nei 1993), which was identified by jModelTest2 (Guindon and Gascuel 2003,

Darriba *et al.* 2012) as the substitution model that best fit our data. We assessed the statistical significance of the F_{ST} and Φ_{ST} values through permutation tests with 10,000 permutations. We also used Fisher's exact tests of differentiation (Raymond and Rousset 1995) with 10,000 permutations to test the null hypothesis of no population structure among the strata, also conducted in Arlequin.

In the eastern Pacific, long-sequence (962 bp) samples from the adjusted data set were pooled and divided into temperate (SST < 25°C) and tropical (SST > 25°C) strata (Fig. 2) based on the definition of temperate and tropical waters in the eastern Pacific (Fiedler and Talley 2006). Mean monthly 0.5°-square SST values for the date and location where each sample was collected were acquired from the Simple Ocean Data Assimilation version 2.2.4 reanalysis (Carton and Giese 2008) for the purpose of binning samples into temperate (SST < 25°C) and tropical (SST > 25°C) strata. Differentiation analyses (F_{ST} , Φ_{ST}) were conducted between temperate and tropical strata to examine the correlation between sea surface temperature and local structure in the eastern Pacific Ocean.

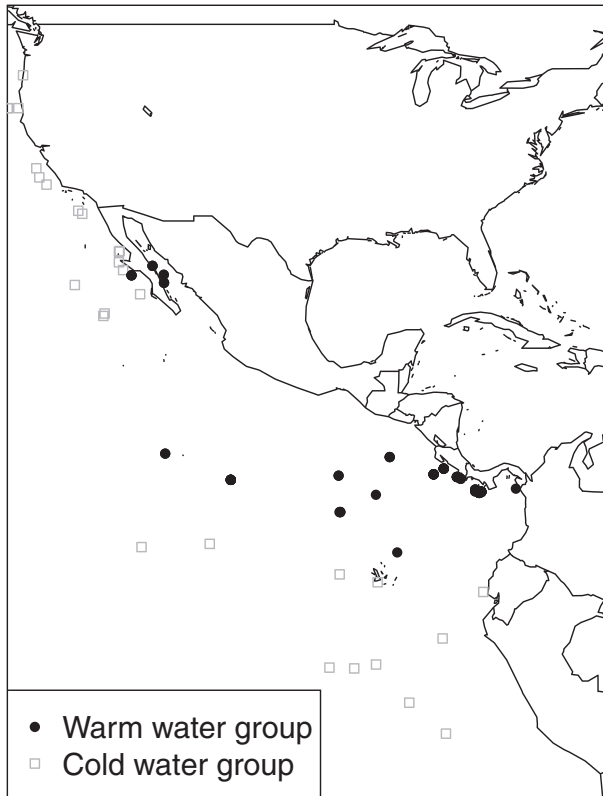


Figure 2. Distribution of temperate and tropical water groups of short-finned pilot whales in the eastern Pacific Ocean, showing the sample stratification used to test for localized structure driven by sea surface temperature. Temperate and tropical water samples were separated at 25°C based on the estimated SST at their collection location on the date they were collected. Circles represent warm water samples while squares represent cold water samples. Only long-sequence (962 bp) samples were used for this analysis. Warm water group $n = 22$, Cold water group $n = 34$.

Finally, a hierarchical AMOVA was conducted on the adjusted data set of short sequences (345 bp) to determine how much of the total variance is described by differences among regions *vs.* among strata within regions. For this analysis, strata outside the Pacific Ocean were excluded. The Pacific Ocean strata were divided into three regions: North/East Pacific (California Current, ETP, and North Japan strata), Central Pacific (Hawai'i, South Japan, and SE Asia strata) and South Pacific. The first two regions correspond to the known geographic distributions of the Naisa and Shiho types (see Results), while the third corresponds with the area where most haplotypes are unknown.

We constructed median-joining networks (MJN) from the full data set using the algorithm of Bandelt *et al.* (1999), implemented in the software package Network 4.6.1.2 (available at <http://www.fluxus-engineering.com/sharenet.htm>), with none of the optional analyses. A median-joining network was first constructed using the 962 bp mtDNA haplotypes sequenced at the SWFSC. A second median-joining network was constructed using combined haplotypes from the SWFSC and GenBank using the shorter portion of the control region (345 bp).

Using previously published sequence data from animals of known morphology (based on skull and body measurements), we identified four haplotypes from known Naisa type animals and one haplotype from known Shiho type animals (Kage 1999). These previously published haplotypes were designated as "known Shiho" or "known Naisa" haplotypes, and are referred to as such in the Results and Discussion. We consider individuals with either a known Shiho or known Naisa haplotype to be either Shiho or Naisa type short-finned pilot whales, respectively. The distribution of all individuals with known Shiho or Naisa haplotypes was mapped to examine the range of each type of animal.

RESULTS

A total of 333 tissue samples from the SWFSC archive were sequenced and analyzed (Fig. 1, Table S1). Using the full 962 bp sequences, we detected 23 unique haplotypes (Table 1) with 24 polymorphic sites. When the sequences generated at SWFSC were truncated to 345 bp for comparison to the GenBank sequences the number of unique haplotypes was reduced to 20. Fourteen haplotypes, representing 152 samples, were obtained from GenBank, representing samples from the North Japan, South Japan, SE Asia, South Pacific, ETP, and western North Atlantic strata. Eight of the haplotypes obtained from GenBank were not present in the samples sequenced at SWFSC, resulting in a total of 28 short (345 bp) haplotypes. A complete list of tissue samples, with their short haplotypes and corresponding long haplotypes, can be found in Table S1.

Using unadjusted short sequence data, the ETP stratum exhibited the largest number of haplotypes in the sample set ($n = 8$, Table 1). There was a large number of haplotypes in the Japanese market samples ($n = 10$), but this group likely include haplotypes from both Naisa and Shiho type short-finned pilot whales. The Hawaiian Islands were unique in that only four haplotypes were found there despite a large sample size ($n = 123$). Of those four haplotypes, two were known Naisa haplotypes, J and K, with J representing 94.3% (116) of the sampled individuals (Table 1). One individual sampled west of Ni'ihau had haplotype C, a haplotype common to the South Pacific and Japanese market samples. Haplotype 12 was found only in the Northwestern Hawaiian Islands (NWHI), which had

Table 1. Short-finned pilot whale haplotype frequencies by strata (Fig. 1), determined using the 345 bp consensus region from SWFSC and GenBank sequences. Haplotypes A through N are labeled according to Oremus *et al.* (2009); additional haplotypes are numbered 2–15. Haplotypes A, D, and E from Oremus *et al.* (2009) were subdivided into seven haplotypes using the 962 bp samples from SWFSC; these haplotypes and their frequencies are shown below the line.

Haplotype	California		Eastern Tropical Pacific		North Japan (Shiho type)		Market Japan		South Japan (Naisa type)		Hawai'i	North Pacific Gyre	South Pacific	SE Asia	Indian Ocean	Western North Atlantic Ocean	Total
	Current																
2	2		14														16
3	1																1
4			1														1
5			2														2
6			8														8
7			1														1
8			3														3
9		1															1
10			5														3
11												1					1
12										5			1				5
13														1			1
14																1	1
15														2		1	1
A													25	2		1	38
B													1				1
C								10					1				1
D					4					1			23			18	34
E	45		96				6										18
F							1										1
G							3										3
H							1										1
I							7		8								15

(Continued)

Table 1. (Continued)

Haplotype	California Current		Eastern Tropical Pacific		North Japan (Shiho type)		Market Japan		South Japan (Naisa type)		Hawai'i		North Pacific Gyre		South Pacific		SE Asia		Indian Ocean		Western North Atlantic Ocean		Total
J							3		1			116											120
K							6		7			1				1							18
L							3																3
M							14		20														34
N							2																2
Total	49	130			4	56		36			123		2		51		4		6		24		486
A1															3 ^a						4 ^a		8
A2																	2				2		6
D1																					2		2
D2																					2		2
E1	2																						2
E2	6																						6
E3	37	95 ^a																					132

^aNote that the total of these haplotypes does not equal the haplotype total above the line, as additional samples from Oremus *et al.* (2009) are included in the totals above line.

14 of the total 123 samples from Hawai'i. Short-finned pilot whales from the ETP and California Current exhibited two shared haplotypes, E and 2. They shared one haplotype with North Japan (E), which until this study was the only known Shiho haplotype in all ocean basins in which short-finned pilot whales occur, and represented 73.8% of the samples in the ETP and 90% of the samples in the California Current (Table 1).

Ten tooth samples from animals with known morphology and sampling location were successfully sequenced (Table 2). The 300 bp segment was compared to the 345 bp haplotypes to identify genetic type for each individual. All Naisa morphotypes had haplotypes matching previously known Naisa haplotypes. Five of the six Shiho morphotypes had haplotype E, the only known Shiho haplotype. The sixth Shiho morphotype had haplotype 5, a haplotype found in the eastern Pacific, which differs from haplotype E by a single substitution.

The SE Asia stratum ($n = 3$) exhibited high haplotypic and nucleotide diversity (Table 3), while North Japan had the lowest, due to small sample size ($n = 1$). All three estimates of diversity were also low in Hawai'i and the eastern Pacific relative to other strata, despite the large samples from these two strata.

Pairwise estimates of differentiation ranged from 0.02 to 0.77 for F_{ST} and 0.00 to 0.88 for Φ_{ST} (Table 4). F_{ST} and Φ_{ST} values were lowest between the ETP and California Current. Values were also lower among SE Asia (*i.e.*, Philippines, Cambodia, and Guam), the South Pacific and New Zealand, and the Indian Ocean, though the small sample size from these areas reduces power to detect differentiation and increases uncertainty in these estimates. F_{ST} , Φ_{ST} , and Fisher's exact tests for differentiation (Table 4, 5) were not significant between the ETP and California Current, nor between South Japan (Naisa) and SE Asia. F_{ST} and Fisher's exact tests for differentiation were nonsignificant among the Indian Ocean, South Pacific and New Zealand, and SE Asia; however, lack of differentiation between these three strata could be due to small samples sizes. It is important to note that we did not calculate differentiation between the North Japan (Shiho) stratum and any other strata due to subsampling that reduced the sample size in that stratum to $n = 1$. However, using the full data set, the North Japan

Table 2. Haplotypes and morphological types (determined from skull measurements) of ten short-finned pilot whales. Note that haplotypes were determined by comparing 300 bp sequences to 345 bp sequences, but there are variable sites in some haplotypes in base pairs 301-345.

ID	Source ID	Morphotype	Haplotype	Sample location
136113	LACM027408	Naisa	J	Hawai'i
136114	LACM054112	Shiho	E	California
136116	LACM054119	Shiho	E	California
136118	LACM054121	Naisa	M	Japan
136119	LACM054122	Naisa	M	Japan
136121	LACM054124	Naisa	M	Japan
136122	LACM054125	Shiho	E	California
136123	LACM054130	Shiho	E	California
136124	LACM054139	Shiho	E ^a	California
136126	LACM054184	Shiho	5	California

^aThis sample is likely haplotype E; it had one ambiguous base pair at a site that was invariable for all other haplotypes.

Table 3. Diversity indices for nine global strata (Fig. 1) calculated using short sequences from the adjusted data set. Standard deviation for haplotype diversity, nucleotide diversity, and θ_H (genetic diversity calculated using observed homozygosity) are shown beneath the values as a \pm value.

	Eastern			South					Western North Atlantic
	California Current	Tropical Pacific	North Japan (Shiho type)	South Japan (Naïsa type)	Hawai'i	Pacific and New Zealand	SE Asia	Indian Ocean	
Sample size	25	35	1	10	29	23	3	5	21
No. haplotypes	4	8	1	4	4	5	3	2	3
No. variable sites	5	9	0	3	8	7	4	3	2
Haplotype diversity (<i>h</i>)	0.297 \pm 0.115	0.560 \pm 0.095	1.000	0.778 \pm 0.091	0.200 \pm 0.10	0.640 \pm 0.065	1.000 \pm 0.272	0.400 \pm 0.237	0.267 \pm 0.120
Nucleotide diversity (π)	0.002 \pm 0.002	0.002 \pm 0.002	0.000	0.004 \pm 0.003	0.002 \pm 0.002	0.004 \pm 0.003	0.008 \pm 0.007	0.004 \pm 0.003	0.008 \pm 0.001
θ_H	0.314 \pm 0.172	0.952 \pm 0.375	NA	2.746 \pm 1.548	0.186 \pm 0.113	1.346 \pm 0.390	NA	0.496 \pm 0.492	0.270 \pm 0.165

Table 4. Pairwise differentiation values for eight geographical strata (Fig. 1) calculated using short sequence data from the adjusted data set. F_{ST} is shown below the diagonal, Φ_{ST} is shown above the diagonal. P -values are shown in parentheses. Significant P -values are in bold.

	California Current $n = 25$	Eastern Tropical Pacific $n = 35$	South Japan (Naisa) $n = 10$	Hawai'i $n = 29$	South Pacific and New Zealand $n = 23$	SE Asia $n = 3$	Indian Ocean $n = 5$	Western North Atlantic $n = 21$
California Current		-0.023 (<0.87526)	0.809 (<0.00001)	0.852 (<0.0001)	0.521 (<0.0001)	0.736 (<0.00079)	0.549 (<0.00059)	0.78 (<0.0001)
Eastern Tropical Pacific	0.02 (<0.17038)		0.777 (<0.00001)	0.823 (<0.00001)	0.479 (<0.0001)	0.685 (<0.00079)	0.47 (<0.0004)	0.712 (<0.0001)
South Japan (Naisa)	0.527 (<0.00001)	0.359 (<0.00001)		0.574 (<0.0001)	0.655 (<0.0001)	0.105 (<0.21602)	0.534 (<0.00218)	0.851 (<0.0001)
Hawai'i	0.755 (<0.00001)	0.609 (<0.00001)	0.561 (<0.00001)		0.744 (<0.0001)	0.639 (<0.0001)	0.738 (<0.00416)	0.882 (<0.0001)
South Pacific and New Zealand	0.336 (<0.00001)	0.404 (<0.00001)	0.296 (<0.0002)	0.59 (<0.00001)		0.48 (<0.0099)	0.202 (<0.09128)	0.58 (<0.0001)
SE Asia	0.567 (<0.00495)	0.331 (<0.02277)	0.082 (<0.27304)	0.67 (<0.00335)	0.082 (<0.27591)		0.157 (<0.36967)	0.816 (<0.00168)
Indian Ocean	0.679 (<0.00001)	0.482 (<0.0001)	0.347 (<0.00515)	0.76 (<0.00001)	0.103 (<0.18879)	0.022 (<0.45956)		0.674 (<0.0002)
Western North Atlantic	0.717 (<0.00001)	0.564 (<0.00001)	0.336 (<0.00001)	0.771 (<0.00001)	0.52 (<0.00001)	0.571 (<0.01119)	0.675 (<0.00059)	

Table 5. Results of Fisher's exact test of differentiation, testing the null hypothesis of nondifferentiation between nine strata of short-finned pilot whale, from the adjusted data set of short sequences. Results shown as permutation values \pm standard deviation. Significant values are in bold. Sample sizes used are the same as Tables 3 and 4.

	Eastern Tropical					South Pacific and		Indian Ocean
	California Current	Pacific	South Japan (Naisa)	Hawai'i	New Zealand	SE Asia		
Eastern Tropical Pacific	0.423 \pm 0.01							
South Japan (Naisa)	0.000 \pm 0.00	0.000 \pm 0.00						
Hawai'i	0.000 \pm 0.00	0.000 \pm 0.00	0.000 \pm 0.00					
South Pacific and New Zealand	0.000 \pm 0.00	0.000 \pm 0.00	0.000 \pm 0.00	0.000 \pm 0.00				
SE Asia	0.005 \pm 0.00	0.008 \pm 0.00	0.136 \pm 0.00	0.004 \pm 0.00	0.080 \pm 0.00			
Indian Ocean	0.000 \pm 0.00	0.000 \pm 0.00	0.009 \pm 0.00	0.000 \pm 0.00	0.274 \pm 0.00	0.643 \pm 0.00		
Western North Atlantic	0.000 \pm 0.00	0.000 \pm 0.00	0.000 \pm 0.00	0.000 \pm 0.00	0.000 \pm 0.00	0.011 \pm 0.00	0.001 \pm 0.00	

(Shiho) stratum is significantly different from all other strata except the ETP and California Current (data not shown).

The hierarchical AMOVA among regions in the Pacific suggested that most genetic variance occurs among regions, with some differentiation among strata within regions (Table S2). Variation among regions accounted for 63% of genetic variance, while approximately 11% of variation occurred among strata within regions and 27% of the variation occurred within strata.

We tested the hypothesis that populations in the eastern Pacific were distributed according to sea surface temperature using full long-sequence data (Fig. 2), and found that the temperate and tropical groups were not significantly differentiated (Fisher's exact $P = 0.7933$), and the magnitude of differentiation between them ($F_{ST} = -0.007$, $P = 0.48$; $F_{ST} = -0.019$, $P = 0.79$) was similar to the comparison between the California Current and ETP strata (Table 4).

The full data set of short-sequence data from SWFSC was compared with previously published data using a median joining network (Fig. 3). The resulting global phylogeography supports the hypothesis that California Current short-finned pilot whales are Shiho type (Oremus *et al.* 2009), with the known Shiho haplotype (E) making up 78% of the stratum. The known Shiho haplotype also represents 73.8% of the ETP stratum. Most of the other ~25% of individuals in the eastern Pacific are only 1–2 base pairs removed from the known Shiho haplotype (*e.g.*, haplotypes

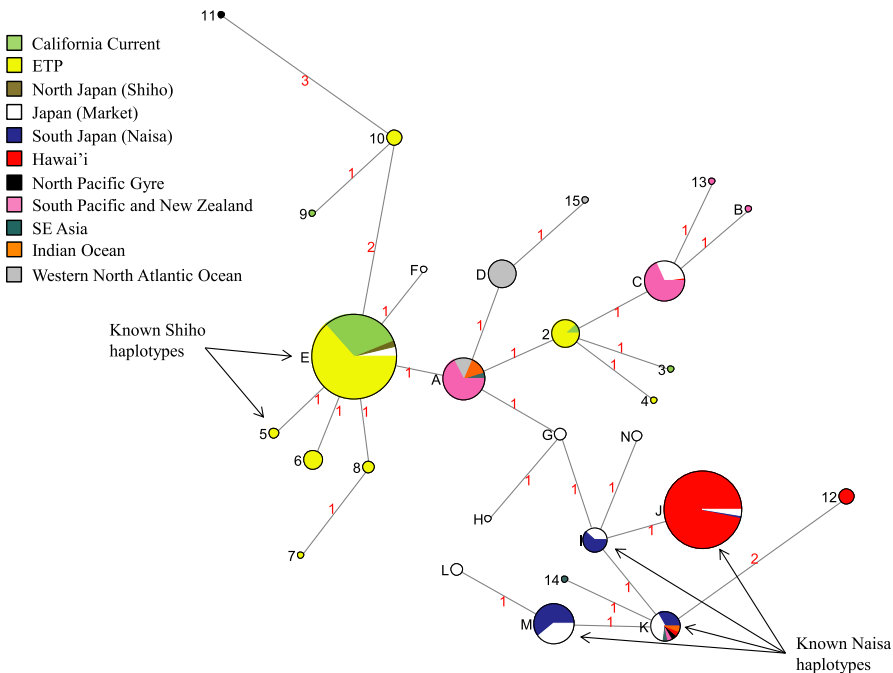


Figure 3. Median joining network created using short (345 bp) sequences from SWFSC and GenBank, including those from Oremus *et al.* (2009). Samples are segregated geographically. Red numbers indicate the number of base pair differences between each haplotype, black labels indicate haplotype. The size of each circle represents the number of individuals with the respective haplotype in the study.

5, 6, 7, 8, 10), and one of these (haplotype 5), was determined to belong to the Shiho morphotype using skulls collected in the eastern Pacific Ocean (Table 2). A few haplotypes found in the eastern Pacific Ocean (haplotypes 2, 3, and 4) were only 2–3 base pairs removed from the known Shiho haplotype (E); it is interesting to note, though, that the path between haplotypes 2–4 and haplotype E, all found in the eastern Pacific Ocean, is through a common haplotype found in the South Pacific and New Zealand stratum. Of the four Hawaiian haplotypes, three were closely related to each other, two of which were known Naisa haplotypes. Most haplotypes from the South Pacific and New Zealand, Indian Ocean, and western North Atlantic strata were grouped together, and clustered with some haplotypes found in the eastern Pacific. Haplotype K, one of the known Naisa haplotypes, was widely dispersed in the Hawai'i, SE Asia, South Pacific and New Zealand, South Japan, market Japan, North Pacific Gyre and Indian Ocean strata. A median-joining network of the longer 962 bp sequences from the SWFSC samples (Fig. S1) shows no change in the overall pattern seen in the dataset of smaller sequences.

The distribution of the known Shiho haplotype extended from northern Japan to the California Current, and throughout the range of pilot whales in the eastern Pacific Ocean (Fig. 4). The known Naisa haplotypes were found in southern Japan, SE Asia, the South Pacific, and Hawai'i. No known Shiho or Naisa haplotypes were found in the western North Atlantic Ocean, but the known Naisa haplotype K was found in the Indian Ocean.

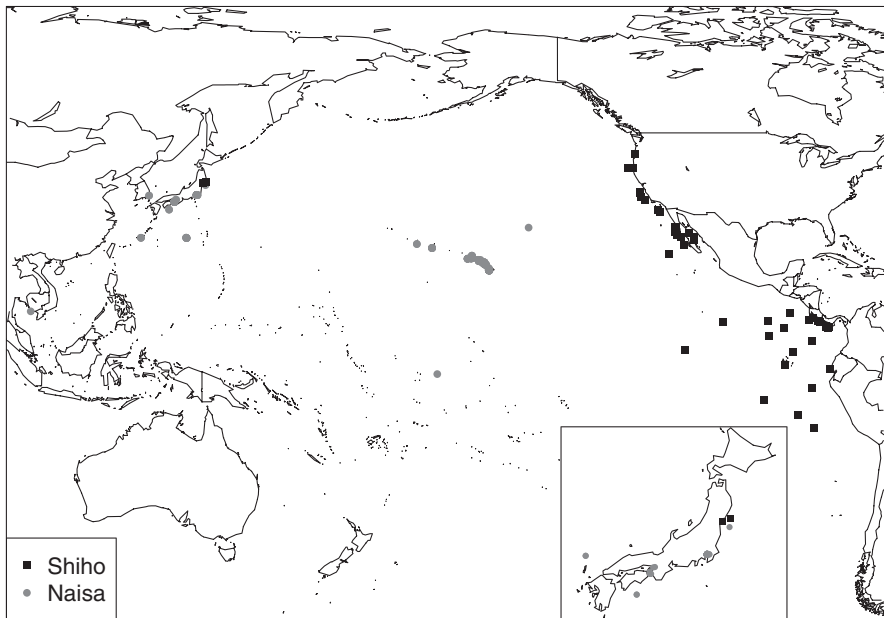


Figure 4. Global distribution of only the known short-finned pilot whale Naisa and Shiho haplotypes, based on previously published DNA sequences from animals of known morphology (skull and body measurements). Shiho type samples are shown as black squares; Naisa type samples are gray circles. Inset: enlarged map of Japan showing details of approximate sample locations (from Oremus *et al.* 2009).

DISCUSSION

Distribution of Shiho and Naisa Types

The taxonomic status of short-finned pilot whales in the Pacific Ocean has been in question since the identification of possible subspecies off of coastal Japan (*e.g.*, Kasuya *et al.* 1988, Oremus *et al.* 2009). While the link between mtDNA haplotypes and morphology is correlative and not causative, it has been used in many taxa to help identify population divergence and speciation (*e.g.*, Avise 1992, Palumbi *et al.* 1997, Oremus *et al.* 2009, Foote *et al.* 2011). The genetic data presented in this paper are not sufficient to resolve the taxonomic question, due to the fact that in highly social cetaceans such as short-finned pilot whales there might be male-mediated gene flow, which mtDNA alone cannot rule out. However, this study provides strong evidence that the distributions of the Shiho and Naisa types are spatio-temporally nonoverlapping in the Pacific Ocean (Fig. 4); this result is consistent with the traditional definition of subspecies as diagnosable geographic forms (Reeves *et al.* 2004).

Our sequences of 10 samples of known morphology provide additional support for the connection between genetic haplotype and morphology in short-finned pilot whales. Notably, this is the first time individuals of known morphology have been sequenced from the eastern Pacific, supporting the conclusion that eastern Pacific short-finned pilot whales are Shiho type animals. Our results also add a new haplotype (5) to those that have been morphologically identified as Shiho haplotypes.

This genetic evidence indicates that the distribution of the two types of pilot whales throughout the Pacific does not follow the north-south distribution found in Japan, therefore the commonly accepted “northern” and “southern” type naming system no longer accurately describes these two types. We recommend that Naisa and Shiho, the historical names used by Yamase (1760) in his original description of these two types, as well as in this study, be re-adopted when referring to the two types of pilot whale in the Pacific Ocean.

Until now the accepted hypothesis has been that the two types of short-finned pilot whale are distributed according to sea surface temperature. In Japan, some evidence suggests that SST may be a proxy for other driving factors (Kasuya *et al.* 1988, Kasuya and Marsh 1984). In the rest of the Pacific Ocean, the morphological and genetic data previously available were limited in number and patchy in their distribution. Using mtDNA we found Naisa type animals in Hawai‘i, but Shiho type animals in both tropical and temperate waters in the eastern Pacific Ocean (*i.e.*, in the California Current and ETP strata). Based on this evidence, and the lack of differentiation found when samples in the eastern Pacific Ocean were stratified according to sea surface temperature, the distribution of these two types does not appear to be driven by sea surface temperature. Rather, it is probable that a combination of ecological, environmental, and possibly social factors drives the distribution of short-finned pilot whales.

The expanse of the oligotrophic central Pacific Ocean could act as a barrier to the dispersal of short-finned pilot whales, thus driving the distribution of the two types. Previous evidence has shown that insular populations of this species prefer slope waters (Baird *et al.* 2013, Mahaffy *et al.* 2015), and therefore movements into more offshore pelagic waters may be limited. Furthermore, while short-finned pilot whales do occur continuously between Hawai‘i and the eastern Pacific Ocean, there is a large area of low density of pilot whales between Hawai‘i and the more productive waters of the ETP (Hamilton *et al.* 2009, Forney *et al.* 2012). However, the presence of

Shiho type pilot whales off of northern Japan and in the eastern Pacific Ocean suggests some past or current gene flow across the ocean basin.

Prey preference may influence the distribution of Shiho and Naisa type pilot whales. The distribution of pilot whales has been shown to be highly correlated with squid, their primary prey (Shane 1995). The two types exhibit differences in dietary preference off Japan (Kubodera and Miyazaki 1993), although in one area the Shiho type has been reported eating the most common squid species (Okutani and McGowan 1969, Sinclair 1992). Little is known of the diet of either type of pilot whale, but it is possible that differences in dietary preference influence the distribution of each type. For example, the geographic range of Humboldt squid (*Dosidicus gigas*), a possible prey item, is similar to the distribution of Shiho type short-finned pilot whales in the eastern Pacific Ocean, while the distribution of Naisa type short-finned pilot whales is similar to that of a closely related squid species, *Sthenoteuthis oualanieensis*, extending eastward from Japan and SE Asia through Hawai'i waters and into the eastern Pacific Ocean (StAAF *et al.* 2010). Both squid species live at 200–700 m, while short-finned pilot whales are known to dive as deep as 800 m (RWB, unpublished data). If Naisa and Shiho type short-finned pilot whales exhibit prey preferences throughout their range, as they do off Japan, it is possible that the extent of the range of each type is influenced by the range of their preferred prey items.

Many of the short-finned pilot whales in this study, including most from the South Pacific, Indian, and western North Atlantic Oceans, do not have known Shiho or Naisa haplotypes. The haplotypes found in these areas may represent one of the two types, which could be elucidated by further genetic analysis. It is also possible that some of these haplotypes represent a third type of short-finned pilot whale. Kasuya *et al.* (1988) suggested the possibility of a second stock of Naisa type short-finned pilot whales, representing a pelagic population of that type separated from the insular stock by the Kuroshio Current. Oremus *et al.* (2009) genetically identified market samples that were otherwise unrepresented in southern Japan, and therefore may belong to a third stock, with a distribution extending into the South Pacific. Our study found a Naisa type haplotype in the Indian Ocean, but failed to find any known Naisa or Shiho haplotypes in the Atlantic Ocean. It remains possible that a third type inhabits part of the South Pacific, Indian, or Atlantic Oceans.

Although short-finned pilot whales are known to occur in the pelagic waters between the ETP and Hawai'i (Hamilton *et al.* 2009), no known genetic or morphological data exist from the area that can be used to determine which type occurs there. In this area and others like it, where genetic and morphological sampling can be difficult, alternative data sources such as acoustic or photo ID data could aid in examining the distribution of the Naisa and Shiho type short-finned pilot whale.

Population Structure and Diversity

Three populations were of primary interest in this analysis: the Hawaiian Islands, the California Current, and the ETP. Short-finned pilot whales in Hawai'i were found to be genetically distinct from all other strata in the Pacific as well as globally, with F_{ST} and Φ_{ST} values ≥ 0.56 , indicating very restricted female-mediated gene flow. For the California Current and ETP strata, samples were not significantly differentiated when stratified either according to geography or SST, suggesting a single population throughout the eastern Pacific Ocean.

There may be further population structure within each of our strata that could be elucidated with more samples, longer DNA sequences or nuclear markers. For

example, 36% of the short-finned pilot whales sampled in the Northwestern Hawaiian Islands shared a single haplotype not found anywhere else. Similarly, both of the samples included in this study from Clipperton Island shared a haplotype unique to that island. Eight of the nine Galapagos samples included in this study share a haplotype only found around those islands. These findings are consistent with the presence of insular populations around Clipperton and the Galapagos Islands, similar to the insular population known to reside in Hawaiian waters (*e.g.*, Baird *et al.* 2012, Mahaffy *et al.* 2015). The SE Asia stratum, which covers a broad geographic area, exhibited the greatest genetic diversity with relatively few samples, suggesting the possibility that greater population structure may be found within that stratum. Our findings highlight the need for higher resolution analyses of population structure within each of the strata included in this study.

Short-finned pilot whales have previously been shown to exhibit strong genetic differentiation between ocean basins, and between populations in Japan (Oremus *et al.* 2009). Here we have shown, using a hierarchical AMOVA, that there is strong differentiation within the Pacific Ocean among the North/East Pacific, Central Pacific, and South Pacific regions. Although sample sizes are small in the Indian Ocean and SE Asia, estimates of F_{ST} and Φ_{ST} differentiation suggest higher levels of gene flow between the Indian Ocean and the western Pacific Ocean than between the eastern and western Pacific Ocean. Although our F_{ST} and Φ_{ST} statistics corroborate previous evidence indicating population differentiation between ocean basins, the occurrence of a shared haplotype among the western North Atlantic Ocean, the Indian Ocean, SE Asia, and the South Pacific Ocean suggests that there may still be continued gene flow among the three ocean basins, or that there may be incomplete lineage sorting due to recent divergence.

Higher haplotypic diversity is found in many of the Pacific Ocean populations than in the western North Atlantic Ocean. Our sample size is small in the Atlantic Ocean, but current evidence from the western North Atlantic suggests that low overall diversity is not an artifact of sample size (Oremus *et al.* 2009, Téllez *et al.* 2014, P. Rosel²). Two differences between the western North Atlantic and Pacific that may influence diversity are immediately notable: the first is the size of the Pacific Ocean compared with the western North Atlantic, and the second is the absence of long-finned pilot whales in the North Pacific Ocean since the 12th century (Kasuya 1975). The two factors combined may have allowed short-finned pilot whales to expand to a presumably greater abundance and into a greater variety of habitats in the Pacific Ocean, thus increasing their haplotypic diversity.

The overall low mtDNA diversity in short-finned pilot whales may inhibit the detection of genetic structure, leading to the conclusion that short-finned pilot whales are a single species globally. However, their complex social structure and regional genetic differentiation suggests that this species may have a higher degree of structure than is immediately evident. Killer whales (*Orcinus* spp.) exhibit similarly low levels of mitochondrial genetic diversity, but analyses of the full mitogenome and multiple nuclear loci (Morin *et al.* 2010, 2015; Foote *et al.* 2011) as well as integrative studies (Foote 2012, Beck *et al.* 2011) have shown that the current species may actually comprise as many as nine geographically, ecologically or socially distinct subspecies or species. The complicated patterns of morphological and genetic

²Personal communication from Patricia Rosel, NOAA NMFS, Southeast Fisheries Science Center, 646 Cajundome Boulevard, Lafayette, LA 70506, 14 July 2014.

diversity in short-finned pilot whales may also be better explained by using mitogenomes and nuclear DNA. Additionally, integrating genetic data with diet data (*e.g.*, stable isotopes) or social structure data (*e.g.*, acoustics, photo ID) could improve our understanding of possible ecological or cultural drivers of the distribution of the Naisa and Shiho types.

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SUPPORTING INFORMATION

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Figure S1. Median joining network created using long (962 bp) sequences from SWFSC samples. Samples are segregated geographically; legend entries are: Asia = Southeast Asia and Guam, ENP = California Current, ETP = Eastern Tropical Pacific, HI = Hawai'i. Red numbers indicate the number of base pair differences between each haplotype, black labels indicate haplotype.

Table S1. Complete list of samples used, including short and long haplotype designations and GenBank accession numbers for sequences obtained from GenBank.

Table S2. Variance components of hierarchical AMOVA to test for regional differences among Pacific short-finned pilot whale populations, with Naisa type, Shiho type, and the South Pacific each considered a separate region. The adjusted data set was divided into strata as for the pairwise and diversity measurements, then grouped into regions.