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Complete mitochondrial genome phylogeographic analysis of killer whales (*Orcinus orca*) indicates multiple species

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Killer whales (*Orcinus orca*) currently comprise a single, cosmopolitan species with a diverse diet. However, studies over the last 30 yr have revealed populations of sympatric "ecotypes" with discrete prey preferences, morphology, and behaviors. Although these ecotypes avoid social interactions and are not known to interbreed, genetic studies to date have found extremely low levels of diversity in the mitochondrial control region, and few clear phylogeographic patterns worldwide. This low level of diversity is likely due to low mitochondrial mutation rates that are common to cetaceans. Using killer whales as a case study, we have developed a method to readily sequence, assemble, and analyze complete mitochondrial genomes from large numbers of samples to more accurately assess phylogeography and estimate divergence times. This represents an important tool for wildlife management, not only for killer whales but for many marine taxa. We used high-throughput sequencing to survey whole mitochondrial genome variation of 139 samples from the North Pacific, North Atlantic, and southern oceans. Phylogenetic analysis indicated that each of the known ecotypes represents a strongly supported clade with divergence times ranging from ~150,000 to 700,000 yr ago. We recommend that three named ecotypes be elevated to full species, and that the remaining types be recognized as subspecies pending additional data. Establishing appropriate taxonomic designations will greatly aid in understanding the ecological impacts and conservation needs of these important marine predators. We predict that phylogeographic mitogenomics will become an important tool for improved statistical phylogeography and more precise estimates of divergence times.

[Supplemental material is available online at http://www.genome.org. The sequence data from this study have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank) under accession nos. GU187153–GU187164, GU187166–GU187219, and HM060332–HM060334.]

Theory and empirical studies have shown ecology to be a driving force in speciation (Schluter 2009). Ancestral populations can subdivide and radiate into novel ecological niches and are then subject to divergent selection and subsequent adaptive divergence, which can lead to reproductive isolation and speciation (Gavrilets and Losos 2009; Schluter 2009). This process of radiation into novel and divergent ecological niches is often characterized by a rapid burst of phenotypic diversification, which then slows as disparate ecological niches become filled (Gavrilets and Losos 2009), and is consistent with phylogenies showing the greatest ecological differences early in a clade's history (Grant and Grant 2008; Losos 2009).

Maintaining high levels of biodiversity by conserving both this process and the resultant genetic and phenotypic variation is an important goal of management bodies (Moritz 2002). Determining units on divergent evolutionary trajectories can facilitate

this, and a number of criteria and concepts have been suggested for defining species, subspecies, or management units. Some concepts are based purely upon genetic criteria such as reciprocal monophyly of mitochondrial DNA (mtDNA) haplotypes and significant divergence of allele frequencies at nuclear loci (e.g., Moritz 1994); others incorporate ecological and phenotypic data to assess "exchangeability" between putative species (e.g., Crandall et al. 2000; De Queiroz 2007). De Queiroz (2007) argued that the many concepts all agree in the basic description of species as independently evolving metapopulations, and that the criteria for defining these species all boil down to different types of supporting evidence.

Phylogenetic analysis using mtDNA is a widely used tool for the genetic component of delineating species (Moritz 1994). The generally rapid rate of mtDNA sequence evolution and lineage sorting (relative to the nuclear genome) facilitate inference of evolutionary patterns (Brown et al. 1982; Avise 1989; Moore 1995), especially for social species with a matrilineal group structure, which is common among terrestrial and oceanic mammals (e.g., Lyrholm and Gyllensten 1998; Nyakaana and Arctander 1999; Okello et al. 2008). Despite the general assumptions and its wide use, however, mtDNA

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sequence can in some cases be an uninformative marker for phylogenetics and species delineation if only portions of the genome are used (Galtier et al. 2009b). Indeed, the mitochondrial "molecular clock" varies widely and has been shown to be especially slow in some taxa, e.g., cetaceans and sharks (Nabholz et al. 2008a,b, 2009; Galtier et al. 2009a). Short sections of the mtDNA genome can therefore be uninformative phylogeographic markers in these taxa (e.g., Hoelzel et al. 2002). A further limitation of traditional mtDNA sequencing that focused on either the control region or cytochrome *b* sequences has been the inability to resolve relationships when a radiation was very rapid. Greater resolution of phylogenies can be achieved by increasing the amount of sequence data (Saitou and Nei 1986; Ruvolo et al. 1991; Cummings et al. 1995; DeFilippis and Moore 2000; Rokas and Carroll 2005).

The advent of highly parallel long-read pyrosequencing technologies with targeted resequencing of large genetic regions from genetically tagged and pooled samples now makes it possible to rapidly and efficiently obtain orders-of-magnitude more sequence data than was previously possible with Sanger sequencing (Meyer et al. 2008). Although whole mitochondrial genomes (mitogenomes) are now available for a number of species, they have typically been generated for deep phylogenetic analysis, and to allow more precise estimates of long divergence times (Arnason et al. 2004, 2008; Xiong et al. 2009). To date, only a few studies have made use of phylogeographic mitogenomics to investigate patterns within a genus or species, and most have involved the use of few genomes, typically from species of medical or economic interest (e.g., Zarowiecki et al. 2007; Carr and Marshall 2008; Torriani et al. 2008). The ability to cost-effectively sequence the entire mitochondrial genome from larger numbers of samples for phylogeographic studies should help to resolve previously intractable polytomies resulting from low levels of sequence divergence or rapid radiations of many more species.

As a case study to investigate the potential of using whole mitochondrial genomes for phylogeography, we have undertaken a study of one such "difficult" species. Killer whales (*Orcinus orca*) are apex predators found in all the world's oceans (Forney and Wade 2006). Currently considered a single species (Rice 1998), local variation in a number of characteristics, including body size, color patterning, social structure, vocalization pattern, and behavior, has led to the recognition of several named killer whale types (often referred to as "ecotypes") (Barrett-Lennard et al. 1996; Ford et al. 1998; Baird 2000; Pitman and Ensor 2003; Deecke et al. 2005; Pitman et al. 2007; Foote et al. 2009; Parsons et al. 2009). In particular, prey specialization appears to be a defining characteristic of these types, with partially or fully sympatric populations having specific, sometimes nonoverlapping prey preferences (e.g., fish vs. marine mammals) (Ford et al. 1998; Saulitis et al. 2000; Pitman and Ensor 2003; Herman et al. 2005; Krahn et al. 2007b). Although ecological specialization is not uncommon (Gavrilets and Losos 2009; Schluter 2009), the fact that killer whales exhibit specialization within an ecosystem that is largely based on social mechanisms is of great interest, suggesting that speciation may have occurred in the absence of physical barriers to gene flow. Many killer whale populations are being negatively impacted by human activities, such as over-fishing and pollution, and such threats are likely to vary substantially between types (e.g., Ross et al. 2000; Ylitalo et al. 2001). Effective management therefore requires the delineation of conservation units (Moritz 1994) within the genus *Orcinus* to facilitate different management strategies.

Despite a worldwide distribution and phenotypic differences among killer whale types, genetic diversity of mtDNA is low, and

the control region and other mtDNA loci have been used with limited success to determine population structure and phylogeography. In a survey of ~1000 bp of the control region from over 100 samples from various locations around the world, Hoelzel et al. (2002) found only 13 haplotypes and no clear pattern of genetic association with ocean basin or type. They concluded that killer whales had gone through a worldwide bottleneck ~145,000–210,000 yr ago (i.e., during the Pleistocene), and that the genetic patterns reflected stochastic distribution of mitochondrial haplotypes following the post-bottleneck expansion, rather than phylogenetic lineages reflecting the evolution of ecotypes. Analysis of an expanded set of mtDNA control region sequences by LeDuc et al. (2008), including 80 samples from three described types in the Southern Ocean around Antarctica, found similar patterns, but also found that two Antarctic types associated with the ice edge were each monophyletic, albeit with very low levels of differentiation. Indeed, levels of differentiation among types worldwide have been marked by only one to six fixed differences and total genetic distances of <0.3% for the most divergent control region lineages. This low level of mtDNA diversity has resulted in only weak inference of phylogeographic patterns and divergence times in killer whales, limiting our ability to understand their evolution and taxonomy. Studies of nuclear microsatellites have begun to clarify population structure within ecotypes, and propose even more recent divergence of regional ecotypes within the last 20,000–40,000 yr (Hoelzel et al. 2007; Pilot et al. 2010).

Killer whales are therefore an ideal candidate species for applying new high-throughput sequencing techniques to allow the production of a highly corroborated mitogenome tree and the testing of hypotheses of the timing of coalescence of killer whale populations (e.g., Hoelzel et al. 2002), with a precision of temporal discrimination not previously possible. Specifically, we test the hypotheses that killer whale ecotypes radiated toward the end of the Pleistocene, that ecotypes diversified regionally within ocean basins, and that mitochondrial haplotypes are stochastically distributed among ecotypes.

Results

Full-length mitochondrial genomes of ~16,390 bp (16,386–16,392 bp) were sequenced and assembled for 143 killer whale samples (Fig. 1) and three other cetacean species (false killer whale *Pseudorca crassidens* and long- and short-finned pilot whales *Globicephala macrorhynchus*, *G. melas*) for use as outgroups. An additional five partial mitochondrial sequences were generated, with one or more gaps in the sequence ranging from 35 bp to ~9 kb. Analysis of eight full mitochondrial sequences that were replicated yielded only two differences (not including polynucleotide repeats), for a sequence error rate of ~0.00076%. These included one sample sequenced in separate U.S. and Danish sequencing facilities as well as intra-lab replication. Mutation rate estimates for the whole mitogenome were 2.6×10^{-3} (1.50–3.83×10^{-3}) substitutions per site per million years for *Orcinus*. This rate is lower than the mean for mammals (3.3×10^{-2}), and similar to rates estimated for other cetaceans (2.3×10^{-3}, extrapolated from third positions only) (Nabholz et al. 2008a).

Previously published mitogenome sequences were combined with sequences generated in this study to estimate the time of divergence for the genus *Orcinus* (Fig. 2A). Within *Orcinus*, 139 mitogenomes (66 distinct haplotypes) were used for further analysis, after removal of duplicates and one poor-quality sequence. Bayesian analysis of the 66 unique haplotypes produced the phylogenetic tree
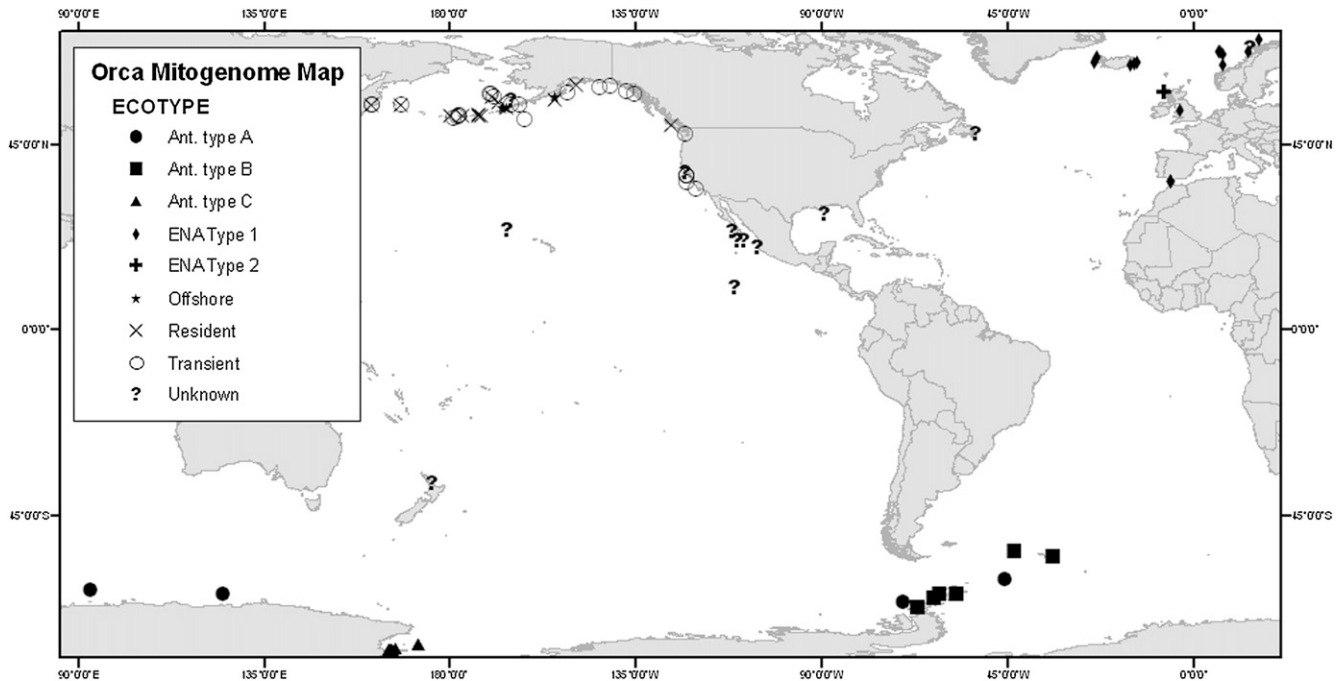
Figure 1. Sample collection locations with indication of type when known.

shown in Figure 2B. In contrast to results based on the control region sequences alone (Hoelzel et al. 2002; LeDuc et al. 2008), no haplotypes were shared between killer whale types or ocean basins. However, animals of unknown type from multiple oceanic regions were grouped in the phylogenetic tree with the Offshore, eastern North Atlantic (ENA) Type 1, Antarctic Type A, and Transient types (Fig. 2B), indicating possible common ancestry of widely separated populations, or, in the case of the one putative Antarctic A type that clusters with eastern North Pacific (ENP) Transient type (predominantly coastal mammal-eating specialist; Ford and Ellis 1999; Baird 2000), potentially an as yet undescribed new killer whale type in the Southern Ocean.

The most striking feature of the phylogenetic tree is the relatively deep divergence of the ENP Transient clade from all others, including two sympatric groups, the Offshore and Resident types. The estimated divergence time is 700,000 yr ago (95% highest posterior density interval [HPDI] = 488,000–960,000), and this clade has 40 fixed differences from all other samples when the single Type A sample is excluded (36 when included). This is 17% of all of the variable sites detected genus-wide. All other types differ from each other by three to 25 fixed differences (Table 1). The time to most recent common ancestor (TMRCA) within Transients is estimated at ~190,000 yr ago, and other types have mean TMRCAs ranging from 59,000 to 117,000 yr ago (Table 2). The Antarctic types (including the single sample from the Gulf of Mexico) together form a clade with a mean TMRCA of ~330,000 yr.

The "Resident" clade includes all fish-eating, coastal Resident types (Baird 2000). The "Offshore" clade includes all ENP Offshore types (a relatively little known type thought to specialize on bony fish and elasmobranchs; Baird 2000; Dahlheim et al. 2008) and other Pacific Ocean samples from off western Mexico and Clipperton Island that were not previously identified as part of the Offshore population. Interestingly, one sample from Newfoundland (western North Atlantic; WNA) also clusters with the ENP

Offshore haplotypes, indicating either an origin of the ENP Offshore and Resident groups from ancestral populations in the North Atlantic, or a remigration of animals in the ENP to the WNA via the Northwest Passage during periods of climate warming.

The "ENA 1-2" clade contains two haplotypes that diverged at approximately the same time as the ENP Offshore and Resident clades. These are from animals sampled near Iceland, Scotland, and England. The Icelandic and English samples were previously categorized as a generalist type (North Atlantic Type 1) that includes individuals specializing on fish and individuals that are thought to predate both fish and mammals (Foote et al. 2009). The Scottish sample was categorized as being from a poorly characterized North Atlantic specialist type (Type 2) and had previously been clustered with Antarctic killer whales based on control region data (Foote et al. 2009). ENA type 2 killer whales were represented by only a single sample, and the sequence contained a large gap (3848 bp). This type has been characterized primarily using museum specimens (Foote et al. 2009), not suitable for long-range PCR. Sequencing methods used to obtain ancient mitogenome sequences (see Ho and Gilbert 2010) may be more suitable for further investigating the phylogenetic relationship between types within the ENA.

The "ENA Type 1" clade clusters a sample from New Zealand with whales from Iceland, Norway, and the Strait of Gibraltar. ENA type 1 has recently been described based on diet and morphology (Foote et al. 2009). Together, the ENA samples cluster closely to the ENP Resident and Offshore types. Samples from both ocean basins appear to have similar levels of haplotypic diversity, so it is unclear whether the ancestral population was in the North Atlantic or the North Pacific, or if both arose from a broader generalist population in lower latitudes that is not yet adequately sampled.

There are three described Antarctic types that differ in diet and morphology (Pitman and Ensor 2003; Pitman et al. 2007). All
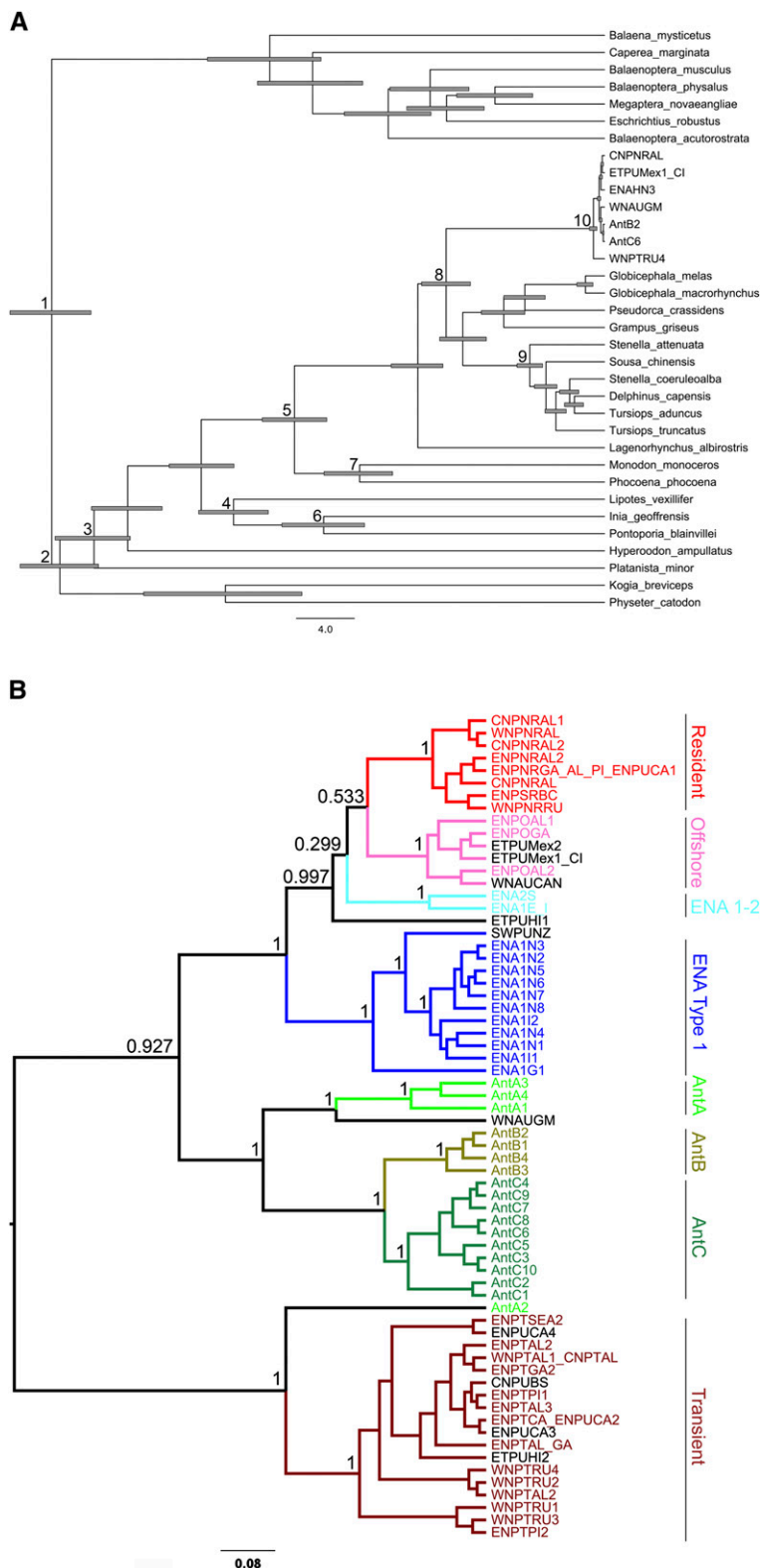
Figure 2. (*A*) Bayesian phylogenetic tree of cetacean phylogeny of whole mitochondrial genomes from the public databases and new species from this study, including 95% highest posterior density interval (HPDI) bars. Nodes with divergence priors are indicated by numbers corresponding to taxonomic groups described in Supplemental Table S5. (*B*) Whole mitochondrial genome phylogeny of 66 unique killer whale haplotypes. Posterior probabilities are indicated for nodes of interest. Whales of known type are indicated in color, and those of unknown type are in black type.

Antarctic samples except one (AntA2) cluster in a monophyletic group that also includes one sample from the Gulf of Mexico in the WNA, and this group is more closely related to all other types in both the ENA and ENP than to the ENP Transients. Two of the three types (B and C) in the Antarctic are also monophyletic, with three fixed differences between types B and C and 24–25 fixed differences between type A (excluding AntA2) and types B and C, respectively. The single sample from the Southern Ocean that does not cluster with the Antarctic types clusters basal to the Transient-type samples, indicating that it may in fact represent a separate population of Transient-like whales in the southern hemisphere. Further sampling in this region is warranted, especially in light of recent observations of a possible fourth pelagic killer whale type in the southern oceans (Jefferson et al. 2007).

Mitochondrial DNA, though useful in phylogeographic studies, has the limitation of being a single, maternally inherited locus. Nuclear markers are needed to obtain data from multiple loci and from both male and female genetic components. Although microsatellites are a poor marker for taxonomic questions, they are the marker of choice for many intraspecific studies, and have been used to study killer whale types. In the North Pacific, previous analysis of 16 nuclear microsatellites has shown that types are genetically very distinct (Hoelzel et al. 2007). Our data from 26 microsatellites genotyped from samples in the North Pacific and Antarctic indicate similarly high levels of differentiation among all killer whale types (Table 3), when divergence is calculated as Hedrick's G'_{ST} to control for intrapopulation levels of diversity (Hedrick 2005). These data are somewhat preliminary due to small sample sizes for three of the killer whale types and no data from ENA types, and though they do not rule out historical or even ongoing low levels of gene flow, they do indicate substantial genetic divergence among types, greater than reported among the majority of comparisons in a recent review of conspecific divergence (Heller and Siegismund 2009). In that review, comparisons that exhibited G'_{ST} levels similar to *sympatric* killer whale types typically involved species where extrinsic barriers to gene flow existed and/or populations exhibited local adaptation (e.g., European wild boars separated by the Alps [Scandura et al. 2008] and locally adapted Atlantic salmon [Dionne et al. 2008]).

Table 1. Fixed differences between killer whale types and oceanic regions

Ecotype comparison	Fixed differences	Variable sites	Haplotypes
North Pacific Transient vs. North Pacific Resident	57	110	27
North Pacific Transient vs. North Pacific Offshore[a]	57	113	25
North Pacific Resident vs. North Pacific Offshore[a]	10	30	14
Antarctic A[b] vs. Antarctic B	24	41	7
Antarctic A[b] vs. Antarctic C	25	55	13
Antarctic B vs. Antarctic C	3	25	14
All Antarctic[c]	5	236	64
Transient[d] vs. non-Transient	40	236	64

Two haplotypes with large sequence gaps (ENASU and AntC7) were not included in calculations.
[a]Offshores include unknown ecotype sample from western Atlantic, Newfoundland (WNAUCAN), and unknown type samples from Mexico and Clipperton Island.
[b]All Antarctic A except sample AntA2, which clusters with the North Pacific Transients (see text).
[c]All Antarctic except sample AntA2, compared with all other complete haplotypes.
[d]All samples in Transient clade (not including AntA2), compared with all other complete haplotypes.

Discussion

Killer whale phylogenetics has been troublesome because of the extremely low levels of diversity found in the mitochondrial control region, so phylogenetic inference was weak or nonexistent (Hoelzel et al. 2002; LeDuc et al. 2008). With highly parallel sequencing technologies, we have developed methods to sequence and assemble whole mitochondrial genomes from representative geographic and ecotype samples to provide strong inference of killer whale evolutionary patterns for the first time. The percent divergence among killer whale types was typically ≥50% higher in the control region than over the whole mitogenome (data not shown), suggesting that simply adding other short segments of mtDNA to the analysis would not significantly improve phylogenetic resolution. Additionally, Bayesian analysis in two phases allowed us to provide a much more accurate and precise date for the most recent common ancestor of all killer whales, and to use that date to estimate divergence times for each of the killer whale types.

Our estimated dates of divergence based on the entire mitogenome are much older than those inferred from short mitochondrial and nuclear loci. Previous studies using mitochondrial control region sequences and microsatellites have inferred that there was a Pleistocene bottleneck ~145,000–200,000 yr ago that reduced variation in killer whale populations globally, followed by recent divergence among the known ecotypes in high-latitude coastal regions. The divergence times were estimated at 20,000–40,000 yr, with wide confidence intervals (Hoelzel et al. 2002, 2007). Using these inferences as our hypotheses, we used whole mitogenomes to infer killer whale evolutionary patterns, and our results indicate much deeper initial separation (either geographic or ecological) between the mammal-eating Transient clade in the North Pacific and a second clade in the Atlantic or lower latitudes ~700,000 yr ago, followed by ecological and/or geographical diversification of the second clade into the present day types at high latitudes, including secondary contact with Transients. These splits between types date from ~150,000–700,000 yr ago rather than 20,000–40,000 yr ago, consistent with species or subspecies level designations. Given the clear lack of phylogenetic information in mitochondrial control region sequences, and the high mutation rates that could cause microsatellites to reach saturation over the time periods that we have inferred from mitogenomes, we believe the mitogenome data provide much stronger support for inference of divergence times.

Recent reviews on subspecies definitions in general have recognized the difficulty in coming up with criteria that will work for defining subspecies and species in all taxa, but they generally agree that data should support discreteness of the subspecies in relation to the remainder of the species and biological significance of the subspecies (Haig et al. 2006). Species concepts are no closer to being universally accepted, but De Queiroz (2007) pointed out that all such concepts agree that species are separately evolving metapopulation lineages, and their delineation is primarily done by accumulation of lines of evidence. Using these criteria, we argue that the combined genetic, morphological, and behavioral evidence of divergence among sympatric types in the high-latitude regions of the North Pacific and Southern Ocean support the recognition of these types as separately evolving metapopulation lineages, and the elevation of three types to species, and several others to subspecies status.

It has previously been suggested that the Southern Ocean B and C types warrant species status on morphological grounds under the biological species concept (BSC), pending confirmation from genetic studies (Pitman and Ensor 2003; Pitman et al. 2007). The genetic data presented here provide such confirmation, demonstrating that the two pagophilic (ice-associated) forms (B and C) are reciprocally monophyletic and form sister taxa substantially divergent from both open-water type A and all other killer whales. Therefore, we recommend that they be designated as distinct species that have diverged from one another for ~150,000 yr and suggest that further analysis of nuclear sequence data should be performed for confirmation.

Our mitogenome data also indicate that the North Pacific Transients should be considered an independent species. Not only are they ecologically and morphologically distinct from other high-latitude killer whales, but genetically they are the most divergent type, diverging from all other killer whale types ~700,000 yr ago. Taxonomic status for the Antarctic A type, North Atlantic types, and North Pacific Resident and Offshore types is less clear due to limited morphological divergence or limited morphological and ecological information, and/or small genetic sample sizes. As such, lines of evidence are not strong for species designation, and

Table 2. Median time to most recent common ancestor (TMRCA) and 95% highest posterior density interval (HPDI) for killer whale types

Clade	TMRCA (million yr)	95% HPDI
Antarctic A	0.117	0.044–0.212
Antarctic B	0.059	0.013–0.136
Antarctic C	0.116	0.051–0.214
Antarctic B and C	0.151	0.067–0.275
All Antarctic[a]	0.331	0.182–0.555
North Pacific Resident	0.080	0.031–0.157
North Pacific Offshore	0.087	0.036–0.166
North Pacific Transient	0.188	0.097–0.312
North Pacific Resident and Offshore	0.177	0.085–0.305
Eastern North Atlantic Type 1[b]	0.168	0.078–0.303
All killer whales	0.702	0.489–0.956

[a]Including single sample from the Gulf of Mexico.
[b]Including single sample from New Zealand.

Table 3. The harmonic mean of standardized differentiation (G'_{ST}) below diagonal, nonstandardized differentiation (G_{ST}) above, based on 26 microsatellite loci

	Resident	Offshore	Transient	Antarctic A	Antarctic B	Antarctic C
Resident	—	0.05	0.07	0.07	0.17	0.15
Offshore	0.19	—	0.04	0.03	0.14	0.12
Transient	0.28	0.15	—	0.02	0.07	0.06
Antarctic A	0.32	0.12	0.10	—	0.06	0.05
Antarctic B	0.56	0.52	0.32	0.30	—	0.02
Antarctic C	0.58	0.53	0.32	0.33	0.11	—

In addition, the long sequences provide both greater power for phylogenetic inference, and greater precision in estimation of divergence times. We expect that, as sequencing technologies continue to allow more samples, more sequence, and lower cost, the application of mitogenomics will become the default approach to phylogeography, as was previously the use of control region and cytochrome *b* sequence analysis.

we recommend subspecies status pending additional nuclear sequence and morphological data.

Low levels of mtDNA variation have limited our ability to resolve evolutionary patterns in killer whales and some other cetacean species. The ability to use whole mitogenome sequences has allowed resolution of phylogeographic patterns. In killer whales these patterns are consistent with historical ecological specialization of small populations (or even single maternal groups) in each region reinforced by either temporary allopatry or in sympatry with social and behavioral isolating mechanisms. Given the typically very small population sizes of killer whale populations, numbering in the low hundreds to thousands, with higher densities in high-latitude coastal regions (Barrett-Lennard and Ellis 2001; Forney and Wade 2006), monophyly might arise quickly in divergent types with little or no female dispersal (Parsons et al. 2009), or dispersal limited to groups with similar vocal and behavioral patterns (Baird et al. 1992; Baird 2000).

The limited sampling in lower latitudes, where diversity is relatively high but density is typically low (Forney and Wade 2006), may mean that we have missed sharing of haplotypes across ocean basins in subtropical and tropical waters, but the patterns for the high latitudes are strongly supported by the more extensive sampling presented here. Most recognized types (except ENA types) have fixed differences in the mitogenomes, indicating independent evolution of each type. The pattern of relatedness among clades is consistent with the independent evolution of feeding specialization in different ocean basins, and apparent diversification of most types within each ocean basin, with the exception of the early separation of Transients from all others.

Type-specific prey specialization largely defines the ecological roles of killer whales and also determines their exposure to human impacts such as fisheries depletion of prey and bioaccumulation of pollutants. Each of these potential species, subspecies, or ecotypes represents a top predator in its ecosystem (or multiple top predators in areas where they are sympatric). As such, and because they are globally distributed, killer whales are critical components of the ocean ecosystems, and represent substantial biological and ecological diversity. Human impacts including over-fishing, persistent organic pollutants, and climate change are already affecting some killer whale populations (e.g., Hickie et al. 2007; Krahn et al. 2007a). Establishing appropriate taxonomic designations is critical for understanding the ecological impacts and conservation needs of these important marine predators, and for maintaining biological diversity and ecosystem health.

As indicated in this killer whale case study, previously reported limitations of using short DNA sequences can be overcome by using whole mitogenomes. High-throughput mitogenomics provides a new tool for intra- and interspecific phylogeography that addresses many of the problems of limited diversity and variable mutation rates and patterns found in short segments of mitochondrial loci.

Methods

DNA extraction and long-range PCR amplification

Skin biopsy samples were obtained from free-ranging killer whales by dart biopsy (e.g., Barrett-Lennard et al. 1996), or from stranded animals. Samples were selected to cover the broadest geographic range as well as genetic and killer whale type diversity. For the mitogenome analysis, most samples were selected from separate collection dates and, when known, identified groups to minimize chances of collecting close relatives or replicate individuals, except in the Southern Ocean, where all samples that had been assigned to one of the three types were included in the sample list (though not all were successfully sequenced). The sequenced sample set included: five (four, after removal of duplicate samples) Antarctic type A (Ant_A), 18 (15) Antarctic Type B (Ant_B), 39 (36) Antarctic type C (Ant_C), five ENP offshore, 11 ENP resident, 17 ENP transient, 12 ENP unknown, 12 eastern tropical Pacific (ETP) unknown, one Gulf of Mexico (unknown), one Newfoundland western Atlantic (unknown), one western South Pacific (New Zealand, unknown), 20 ENA Type 1, one ENA Type 2. In the Antarctic sample sets, we used all available samples, including one to four individuals each from 11 social groups of type C, and one to seven individuals from six social groups of type B whales. In the ETP, multiple (two to four) samples from four social groups were included in the analysis. All other samples are thought to be single samples from a social group. Sample collection locations and types are shown in Figure 1, and additional sample information is shown in Supplemental Table S1.

DNA was extracted using a variety of common extraction methods, including silica-based filter membranes (Qiagen), standard phenol/chloroform extraction (modified from Sambrook et al. 1989), and lithium chloride (Gemmell and Akiyama 1996). Outgroup sequences were obtained from the NCBI GenBank or generated for this study (Supplemental Table S2).

PCR primers were designed from alignment of published whole mitochondrial genomes of other cetacean species, and partial mitochondrial genome sequences of killer whales to amplify the whole mitogenome in two to five overlapping fragments (Supplemental Table S3). PCR conditions for long-range amplification are given in Supplemental Table S3.

454 Life Sciences (Roche) sequencing

PCR products were quantified using Nanodrop (Thermo Scientific) or QuantIt Pico Green (Invitrogen) and pooled in equimolar concentrations for each sample. Samples were made into shotgun sequencing libraries following the manufacturer's instructions (454 Life Sciences [Roche]). Sample pools were grouped into sets, and within each sample set individual libraries were made to contain a different multiplexing identifier (MID) allowing for the combining of the libraries prior to emulsion PCR. Libraries were tagged for multiplexing according to the manufacturer's instructions (454 Life Sciences [Roche]) or Meyer et al. (2008). Sequencing libraries

were quantified by qPCR (Meyer et al. 2007) and pooled at equimolar concentrations. Library pools were divided among regions on GS FLX and Titanium sequencing runs. Sequences for 66 unique killer whale mitogenome haplotypes were deposited in GenBank (accession nos. GU187153–GU187164, GU187166–GU187219), as well as new mitogenome sequences for three outgroup species (accession nos. HM060332–HM060334). All accession numbers used for analysis are listed in Supplemental Table S2.

Microsatellites

A set of 26 microsatellite loci was used to genotype all Antarctic samples and samples collected across the northern North Pacific for population analysis and identification of samples representing duplicate biopsy sampling of the same individual. Forty-four samples were intentionally genotyped in duplicate to estimate error rates, and an additional 15 samples were found to be duplicate samplings of the same individual. One of each pair of unintentional duplicates was removed prior to statistical analysis. Sample sizes (after removal of duplicates) were: Transient, 126; Resident, 245; Offshore, six; Antarctic A, eight; Antarctic B, 15; Antarctic , 42. To test for genotyping errors, we compared replicated genotypes across all loci for replicated samples, and found a per-allele error rate of 0.2%, which is in the low range for published studies (Morin et al. 2010). Population differentiation was calculated using G_{ST} and Hedrick's G'_{ST} to control for the effect of heterozygosity, using the program SMOGD (Crawford 2010). The approximate harmonic mean (H) was calculated from the mean and variance across loci using the equation

$$H \sim 1/[(1/A) + \text{var}(D)(1/A)^3]$$

where A = average divergence across loci and var(D) = variance of divergence across loci (SMOGD website, http://www.ngcrawford.com/django/jost/).

Sequence assembly and phylogenetic analysis

Sequence reads for each sample were sorted by tag sequences, and a single sample was assembled de novo into a single 16,388-bp contig using 454 de novo Assembler software (Roche Applied Science). The consensus sequence and assembly reads were exported as an ACE file and edited with Consed (Gordon et al. 1998), and used as the reference sequence for all subsequent assemblies using the 454 Reference Mapper software (Roche Applied Science). Consensus sequences were aligned in Sequencher (v.4.7, Gene Codes Corporation) or GENEIOUS (Biomatters Ltd), and ambiguities in polynucleotide repeats were individually checked in the 454 Reference Mapper assembly viewer and edited in Sequencher or GENEIOUS. For a region of between nine and 14 Cs in a row (positions 1130–1144 in the original alignment), and another region of seven to eight As in a row (positions 5210–5217), the assembly was unreliable, so the regions were shortened to a fixed set of nine Cs and seven As, respectively, for phylogenetic analysis to avoid introducing potentially erroneous variation. Eight samples were sequenced twice and analyzed for differences between replicates. Sequence alignments of other cetacean sequences and killer whale sequence were performed using Clustal v2.0.4 (Larkin et al. 2007). A figure showing variable sites is shown in Supplemental material S4.

Neighbor-joining trees (MEGA4; Tamura et al. 2007) were constructed initially to select a subset of samples that represented the diversity in the killer whale clades. Bayesian phylogenetic trees and estimates of time since divergence of clades were conducted using BEAST v1.4.8 (Drummond and Rambaut 2007). The HKY

nucleotide substitution model was used, with relaxed clock and a Yule speciation process. We performed two sequential analyses to first estimate divergence times for the genus *Orcinus*, then for types within killer whales. In the first analysis, posterior distributions for divergence times of other cetaceans were used to estimate the divergence time for killer whales, using a set of seven samples representing each of the killer whale clades (Supplemental Table S5). The posterior distribution of divergence time for *Orcinus* from this analysis was then used as a prior distribution with all unique haplotypes to generate the killer whale phylogenetic tree and divergence time estimates for types. The phase I analysis used a burn-in period of 100,000 Markov chain Monte Carlo (MCMC) steps, 100 million total MCMC steps, and samples taken every 1000 steps. Phase II analysis was identical, except that the burn-in period was 80,000 and 80 million MCMC steps were used. Acceptable mixing and convergence to the stationary distribution were checked by visual inspection of posterior samples. Effective sample sizes were 1701 for *Orcinus* in the phase I analysis, 2700–5500 for type clades in the phase II analysis.

Acknowledgments

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S1:

Sample information. Ecotype is based on observed group feeding behavior, geographic location, morphological characteristics, and/or group size; when an ecotype has not been designated, feeding behavior of the whales is indicated if known. Mitogenome label are unique for each mitogenome haplotype as shown in the phylogenetic tree, and are abbreviations meant to convey information about ocean basin, ecotype, and geographic region(s) represented by the samples with that haplotype. Control region (CR) haplotype labels follow the convention of Hoelzel *et al.* (1, 2) and LeDuc *et al.* (3).

Sample ID	Ecotype or feeding information	Mitogenome haplotype	Mitogenome label	CR haplotype	CR haplotype label	Geographic region
Ant_A_17220	Ant_A	MtGen_1	AntA1	4	ANTA4	ANTARCTICA
Ant_A_17223	Ant_A	MtGen_2	AntA2	1	ANTA1	ANTARCTICA
Ant_A_17225	Ant_A	MtGen_3	AntA3	4	ANTA4	ANTARCTICA
Ant_A_17234	Ant_A	MtGen_4	AntA4	5	ANTA5	ANTARCTICA
Ant_B_17230	Ant_B	MtGen_5	AntB1	10	ANTB1	ANTARCTICA
Ant_B_31884	Ant_B	MtGen_6	AntB2	12	ANTB3	ANTARCTIC WATERS
Ant_B_31885	Ant_B	MtGen_6	AntB2	12	ANTB3	ANTARCTIC WATERS
Ant_B_32005	Ant_B	MtGen_7	AntB3	10	ANTB1	ANTARCTICA, WEDDELL SEA, NEAR DEVIL IS.
Ant_B_32006	Ant_B	MtGen_7	AntB3	10	ANTB1	ANTARCTICA, WEDDELL SEA, NEAR DEVIL IS.
Ant_B_32007	Ant_B	MtGen_7	AntB3	10	ANTB1	ANTARCTICA, WEDDELL SEA, NEAR DEVIL IS.
Ant_B_32008	Ant_B	MtGen_7	AntB3	10	ANTB1	ANTARCTICA, WEDDELL SEA, NEAR DEVIL IS.
Ant_B_32009	Ant_B	MtGen_8	AntB4	11	ANTB2	ANTARCTICA
Ant_B_32010	Ant_B	MtGen_8	AntB4	11	ANTB2	ANTARCTICA
Ant_B_32012	Ant_B	MtGen_8	AntB4	11	ANTB2	ANTARCTICA
Ant_B_32013	Ant_B	MtGen_8	AntB4	11	ANTB2	ANTARCTICA
Ant_B_32015	Ant_B	MtGen_8	AntB4	11	ANTB2	ANTARCTICA
Ant_B_40882	Ant_B	MtGen_8	AntB4	11	ANTB2	FALKLAND IS., SOUTH GEORGIA
Ant_B_40883	Ant_B	MtGen_8	AntB4	11	ANTB2	FALKLAND IS., SOUTH GEORGIA
Ant_B_53865	Ant_B	MtGen_8	AntB4	11	ANTB1	FALKLANDS
Ant_C_26617	Ant_C	MtGen_10	AntC1	13	ANTC1	ANTARCTICA
Ant_C_26618	Ant_C	MtGen_10	AntC1	13	ANTC1	ANTARCTICA
Ant_C_26621	Ant_C	MtGen_10	AntC1	13	ANTC1	ANTARCTICA

Ant_C_26614	Ant_C	MtGen_9	AntC10	13	ANTC1	ANTARCTICA
Ant_C_26615	Ant_C	MtGen_9	AntC10	13	ANTC1	ANTARCTICA
Ant_C_26616	Ant_C	MtGen_9	AntC10	13	ANTC1	ANTARCTICA
Ant_C_26619	Ant_C	MtGen_11	AntC2	13	ANTC1	ANTARCTICA
Ant_C_26620	Ant_C	MtGen_12	AntC3	13	ANTC1	ANTARCTICA
Ant_C_26623	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, ROSS SEA
Ant_C_26624	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, ROSS SEA
Ant_C_26625	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, ROSS SEA
Ant_C_45799	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARTICA, MCMURDO SOUND, OFF ROSS ISLAND
Ant_C_45802	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARTICA, MCMURDO SOUND, OFF ROSS ISLAND
Ant_C_45803	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARTICA, MCMURDO SOUND, OFF ROSS ISLAND
Ant_C_45810	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARTICA, MCMURDO SOUND, OFF ROSS ISLAND
Ant_C_45813	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARTICA, MCMURDO SOUND, OFF ROSS ISLAND
Ant_C_53851	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, MCMURDO SOUND
Ant_C_53855	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, MCMURDO SOUND
Ant_C_53856	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, MCMURDO SOUND
Ant_C_53858	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, MCMURDO SOUND
Ant_C_53859	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, MCMURDO SOUND
Ant_C_53860	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, MCMURDO SOUND
Ant_C_53861	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, MCMURDO SOUND
Ant_C_53862	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, MCMURDO SOUND
Ant_C_53864	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, MCMURDO SOUND
Ant_C_53866	Ant_C	MtGen_13	AntC4	14	ANTC2	ANTARCTICA, MCMURDO SOUND
Ant_C_26626	Ant_C	MtGen_14	AntC5	13	ANTC1	ANTARCTICA, ROSS SEA
Ant_C_26627	Ant_C	MtGen_15	AntC6	15	ANTC3	ANTARCTICA, ROSS SEA
Ant_C_26628	Ant_C	MtGen_15	AntC6	15	ANTC3	ANTARCTICA, ROSS SEA
Ant_C_26629	Ant_C	MtGen_15	AntC6	15	ANTC3	ANTARCTICA, ROSS SEA
Ant_C_45800	Ant_C	MtGen_16	AntC7	14	ANTC2	ANTARTICA, MCMURDO SOUND, OFF ROSS ISLAND
Ant_C_45801	Ant_C	MtGen_17	AntC8	15	ANTC3	ANTARTICA, MCMURDO SOUND, OFF ROSS ISLAND

Ant_C_45806	Ant_C	MtGen_18	AntC9	14	ANTC2	ANTARTICA, MCMURDO SOUND, OFF ROSS ISLAND
Ant_C_45809	Ant_C	MtGen_18	AntC9	14	ANTC2	ANTARTICA, MCMURDO SOUND, OFF ROSS ISLAND
Ant_C_53852	Ant_C	MtGen_18	AntC9	14	ANTC2	ANTARCTICA, MCMURDO SOUND
Ant_C_53857	Ant_C	MtGen_18	AntC9	14	ANTC2	ANTARCTICA, MCMURDO SOUND
CNPNR_AL_35320	RESIDENT	MtGen_19	CNPNRAL	35	ENPAR	AK, ALEUTIAN IS., NW OF SEGUAM IS., NO.2
CNPSR_AL_28512	RESIDENT	MtGen_21	CNPNRAL1	32	ENPSR, ENAUK4, WNAN	ALEUTIAN PASSES,AMLIA ISLAND (SOUTH SIDE-EAST)
CNPSR_AL_28548	RESIDENT	MtGen_22	CNPNRAL2	32	ENPSR, ENAUK4, WNAN	ALASKA, NMML DART02 CRUISE,S. SEGUAM PASS
NPBSU_7968	UNKNOWN	MtGen_57	CNPUBS	35	ENPAR	BERING SEA
ENA_EM_14	Type 1	MtGen_23	ENA1E_I	32	ENPSR, ENAUK4, WNAN	River Mersey, England
ENA_IU_19	UNKNOWN	MtGen_23	ENA1E_I	32	ENPSR, ENAUK4, WNAN	W. Iceland
ENA_IU_23	UNKNOWN	MtGen_23	ENA1E_I	32	ENPSR, ENAUK4, WNAN	W. Iceland
ENA_GT_13	Type 1	MtGen_24	ENA1G1	21	ENANo1,ENAI2, WSPNZ1	Strait of Gibraltar
ENA_GT_55	Type 1	MtGen_24	ENA1G1	21	ENANo1,ENAI2, WSPNZ1	Strait of Gibraltar
ENA_GT_56	Type 1	MtGen_24	ENA1G1	21	ENANo1,ENAI2, WSPNZ1	Strait of Gibraltar
ENA_GT_57	Type 1	MtGen_24	ENA1G1	21	ENANo1,ENAI2, WSPNZ1	Strait of Gibraltar
ENA_GT_60	Type 1	MtGen_24	ENA1G1	21	ENANo1,E	Strait of Gibraltar

					NAI2, WSPNZ1	
ENA_GT_8	Type 1	MtGen_24	ENA1G1	21	ENANo1,ENAI2, WSPNZ1	Strait of Gibraltar
ENA_IH_12	Type 1	MtGen_26	ENA1I1	21	ENANo1,ENAI2, WSPNZ1	SW Iceland
ENA_IH_20	Type 1	MtGen_26	ENA1I1	UK1	ENAI1, ENAUK1	SW Iceland
ENA_IH_38993	Type 1	MtGen_26	ENA1I1	UK1	ENAI1, ENAUK1	SE Iceland
ENA_IH_6	Type 1	MtGen_27	ENA1I2	21	ENANo1,ENAI2, WSPNZ1	SE Iceland
ENA_NH_28	Type 1	MtGen_25	ENA1N1	21	ENANo1,ENAI2, WSPNZ1	Tysfjord, Norway
ENA_NH_26567	Type 1	MtGen_28	ENA1N2	23	ENANo2	LOFOTEN IS., NORWAY
ENA_NH_26570	Type 1	MtGen_29	ENA1N3	23	ENANo2	LOFOTEN IS., NORWAY
ENA_NH_39	Type 1	MtGen_30	ENA1N4	21	ENANo1,ENAI2, WSPNZ1	Tysfjord, Norway
ENA_NH_42	Type 1	MtGen_31	ENA1N5	No2	ENANo2	Northern Norway
ENA_NH_49	Type 1	MtGen_31	ENA1N5	No2	ENANo2	Northern Norway
ENA_NH_50	Type 1	MtGen_31	ENA1N5	No2	ENANo2	Northern Norway
ENA_NH_46	Type 1	MtGen_32	ENA1N6	No2	ENANo2	Northern Norway
ENA_NH_47	Type 1	MtGen_33	ENA1N7	No2	ENANo2	Northern Norway
ENA_NH_9	Type 1	MtGen_34	ENA1N8	No2	ENANo2	Northern Norway
ENA_SU_10	Type 2	MtGen_35	ENA2S	UK2	ENAUK2	Harris, Scotland
ENPNR_AL_47869	RESIDENT	MtGen_43	ENPNRAL2	35	ENPAR	BERING SEA
ENPNR_AL_28545	RESIDENT	MtGen_42	ENPNRGA_AL_PI_ENPUCA1	35	ENPAR	ALASKA, NMML DART02 CRUISE,UNALGA PASS
ENPNR_GoA_	RESIDENT	MtGen_42	ENPNRGA_AL_PI_E	35	ENPAR	BERING SEA (AK, USA)

28420			NPUCA1			
ENPNR_PI_47867	RESIDENT	MtGen_42	ENPNRGA_AL_PI_ENPUCA1	35	ENPAR	BERING SEA
ENPR_GoA_43606	RESIDENT	MtGen_42	ENPNRGA_AL_PI_ENPUCA1	35	ENPAR	AK, GULF OF ALASKA
ENPU_CAL_5013	UNKNOWN	MtGen_42	ENPNRGA_AL_PI_ENPUCA1	60		CALIFORNIA GILLNET
ENPO_AL_28521	OFFSHORE	MtGen_45	ENPOAL1	28	ENPO, ENAG	UNALASKA ISLAND(KORIGA PT) NE OF MAKUSHIN BAY
ENPO_AL_28522	OFFSHORE	MtGen_45	ENPOAL1	28	ENPO, ENAG	UNALASKA ISLAND(KORIGA PT) NE OF MAKUSHIN BAY
ENPO_AL_35308	OFFSHORE	MtGen_46	ENPOAL2	28	ENPO, ENAG	AK, ALEUTIAN IS., NW OF PRIEST ROCK, UNALASKA BAY
ENPO_GoA_31872	OFFSHORE	MtGen_47	ENPOGA	28	ENPO, ENAG	AK,GULF OF ALASKA,TRINITY ISLANDS #2
ENPO_GoA_31873	OFFSHORE	MtGen_47	ENPOGA	28	ENPO, ENAG	AK,GULF OF ALASKA,TRINITY ISLANDS #2
ENPSR_BC_74422	RESIDENT	MtGen_48	ENPSRBC	32	ENPSR, ENAUK4, WNAN	British Columbia, Canada
ENPGAT2_AL_67939	TRANSIENT	MtGen_36	ENPTAL_GA	53	ENPT2, GAT2, NT2	USA, AK, ALEUTIANS, SE OF CAPE LAZAREF
ENPGAT_AL_28412	TRANSIENT	MtGen_38	ENPTAL2	59	GAT	BERING SEA (AK, USA)
ENPGAT_AL_43922	TRANSIENT	MtGen_39	ENPTAL3	59	GAT	IFS
ENPT_CAL_45395	TRANSIENT	MtGen_49	ENPTCA_ENPUCA2	59	GAT	CA,SANTA BARBARA CHANNEL
ENPU_CAL_51436	TRANSIENT	MtGen_49	ENPTCA_ENPUCA2	59	GAT	CA, MBNMS
ENPGAT2_GoA_43548	TRANSIENT	MtGen_37	ENPTGA1	53	ENPT2, GAT2, NT2	AK
ENPGAT_GoA_28403	TRANSIENT	MtGen_40	ENPTGA2	59	GAT	NORTH PACIFIC, GULF OF ALASKA (AK, USA)
ENPT_GoA_4	TRANSIENT	MtGen_40	ENPTGA2	59	GAT	AK

3570						
ENPGAT_PI_5 7939	TRANSIENT	MtGen_41	ENPTPI1	59	GAT	BERING SEA, SW SIDE OF ST. PAUL IS.
ENPNT1_PI_4 7862	TRANSIENT	MtGen_44	ENPTPI2	71	ENPT1, NT1	BERING SEA
ENPNT1_PI_5 7943	TRANSIENT	MtGen_44	ENPTPI2	71	ENPT1, NT1	BERING SEA, NW OF ST. GEORGE IS.
ENPGAT2_SE A_44007	TRANSIENT	MtGen_36	ENPTAL_GA	53	ENPT2, GAT2, NT2	AK
ENPT_SEA_2 8487	TRANSIENT	MtGen_50	ENPTSEA2	52	ENPT1, WCT	ALASKA, SITKA, OFFSHORE
ENPT_SEA_4 8592	TRANSIENT	MtGen_50	ENPTSEA2	52	ENPT1, WCT	WA, HOOD CANAL
ENPU_CAL_5 1434	TRANSIENT	MtGen_51	ENPUCA3	59	GAT	CA, MBNMS
ENPU_CAL_5 1435	UNKNOWN	MtGen_51	ENPUCA3	59	GAT	CA, MBNMS
ENPU_CAL_6 004	UNKNOWN	MtGen_52	ENPUCA4	52	ENPT1, WCT	CA, SONOMA CO., BODEGA BAY DUNES
ETPU_HI_305 25	UNKNOWN	MtGen_54	ETPUHI1	6	ANT6	HAWAII
ETPU_HI_305 26	UNKNOWN	MtGen_55	ETPUHI2	62		HAWAII
ETPU_CI_119 79	OFFSHORE	MtGen_53	ETPUMex1_CI	28	ENPO, ENAG	CLIPPERTON IS.
ETPU_CI_119 81	OFFSHORE	MtGen_53	ETPUMex1_CI	28	ENPO, ENAG	CLIPPERTON IS.
ETPU_Mex_3 7946	OFFSHORE	MtGen_53	ETPUMex1_CI	28	ENPO, ENAG	MEXICO
ETPU_Mex_1 5867	OFFSHORE	MtGen_56	ETPUMex2	28	ENPO, ENAG	MEXICO
ETPU_Mex_1 8008	OFFSHORE	MtGen_56	ETPUMex2	28	ENPO, ENAG	MEXICO
ETPU_Mex_1 8093	OFFSHORE	MtGen_56	ETPUMex2	28	ENPO, ENAG	MEXICO
ETPU_Mex_1 8094	OFFSHORE	MtGen_56	ETPUMex2	28	ENPO, ENAG	MEXICO

ETPU_Mex_3 7948	OFFSHORE	MtGen_56	ETPUMex2	28	ENPO, ENAG	MEXICO
ETPU_Mex_3 7949	OFFSHORE	MtGen_56	ETPUMex2	28	ENPO, ENAG	MEXICO
ETPU_Mex_3 7951	OFFSHORE	MtGen_56	ETPUMex2	28	ENPO, ENAG	MEXICO
SWPU_NZ_28 485	UNKNOWN	MtGen_58	SWPUNZ	21	ENANo1,E NAI2, WSPNZ1	NEW ZEALAND, COROMANDEL
WNAU_CA_47 6	UNKNOWN	MtGen_59	WNAUCAN	32	ENPSR, ENAUK4, WNAN	CANADA, NEWFOUNDLAND, BAULINE
WNAU_GM_3 9127	UNKNOWN	MtGen_60	WNAUGM	16		GULF OF MEXICO
WNPSR_AL_5 7912	RESIDENT	MtGen_65	WNPNRAL	32	ENPSR, ENAUK4, WNAN	WESTERN ALEUTIANS, W OF KISKA IS.
WNPSR_AL_5 7921	RESIDENT	MtGen_65	WNPNRAL	32	ENPSR, ENAUK4, WNAN	WESTERN ALEUTIANS, N SIDE OF SEMISOPOCHNOI IS.
WNPSR_RU_4 0252	RESIDENT	MtGen_66	WNPNRRU	32	ENPSR, ENAUK4, WNAN	RUSSIA, KAMCHATKA PENINSULA, KARAGINSKY IS.
WNPSR_RU_6 2387	RESIDENT	MtGen_66	WNPNRRU	32	ENPSR, ENAUK4, WNAN	RUSSIA, COMMANDER, MEDNY ISLAND
CNPNT2_AL_ 35323	TRANSIENT	MtGen_20	WNPTAL1_CNPTAL	57	ENPT2, GAT2, NT2	AK, ALEUTIAN IS., NW OF ULAK IS., NO.2
CNPNT2_AL_ 57906	TRANSIENT	MtGen_20	WNPTAL1_CNPTAL	57	ENPT2, GAT2, NT2	CENTRAL ALEUTIANS, W OF TANAGA BAY, TANAGA IS.
WNPAT1_AL_ 57919	TRANSIENT	MtGen_20	WNPTAL1_CNPTAL	63	AT1	WESTERN ALEUTIANS, S OF SEGULA IS.
WNPNT2_AL_ 57908	TRANSIENT	MtGen_63	WNPTAL2	57	ENPT2, GAT2, NT2	WESTERN ALEUTIANS, NEAR SEA LION RK, BETWEEN KISKA
WNPT_RU_40	TRANSIENT	MtGen_61	WNPTRU1	71	NT1	RUSSIA

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WNPAT1_RU_40249	TRANSIENT	MtGen_62	WNPTRU2	63	AT1	RUSSIA, KAMCHATKA PENINSULA, OLUTYOSKY BAY, GOVENA
WNPNT_RU_62239	TRANSIENT	MtGen_64	WNPTRU3	37	NT4	RUSSIA
WNPT_RU_68629	TRANSIENT	MtGen_67	WNPTRU4	64	NT3	RUSSIA, COMMANDER, MEDNY IS.

Mitogenome label abbreviations

ocean basin		ecotype		geographic region
ENP	eastern North Pacific	R	Resident	see the expanded geographic region info in the table.
CNP	central North Pacific	O	Offshore	see the expanded geographic region info in the table.
WNP	western North Pacific	T	Transient	see the expanded geographic region info in the table.
ETP	eastern Tropical Pacific	A	Antarctic type A	see the expanded geographic region info in the table.
ENA	eastern North Atlantic	B	Antarctic type B	see the expanded geographic region info in the table.
WNA	western North Atlantic	C	Antarctic type C	see the expanded geographic region info in the table.
Ant	Antarctic	1	ENA fish specialist	see the expanded geographic region info in the table.
		2	ENA poorly known type	see the expanded geographic region info in the table.

1. Hoelzel AR, Hey J, Dahlheim ME, Nicholson C, Burkanov V, & Black N (2007) *Mol Biol Evol* **24,** 1407-1415.

2. Hoelzel AR, Natoli A, Dahlheim ME, Olavarria C, Baird RW, & Black NA (2002) *Proceedings of the Royal Society of London, B* **269,** 1467-1473.

3. LeDuc RG, Robertson KM, & Pitman RL (2008) *Biology letters* **4,** 426-429.

S2:
Mitogenome sequences used for phylogenetic analysis (Figure 1)

Species	Accession No.
Balaena mysticetus	NC005268
Balaenoptera acutorostrata	NC005271
Balaenoptera musculus	NC001601
Balaenoptera physalus	NC001321
Caperea marginata	NC005269
Delphinus capensis	EU557094
Eschrichtius robustus	NC005270
Grampus grisseus	EU557095
Hyperoodon ampullatus	NC005273
Inia geoffrensis	NC005276
Kogia breviceps	NC005272
Lagenorhynchus albirostris	NC005278
Lipotes vexillifer	NC007629
Megaptera novaeanglia	NC006927
Monodon monoceros	NC005279
Phocoena phocoena	NC005280
Physeter catadon	NC002503
Platanista minor	NC005275
Pontoporia blainvillei	NC005277
Sousa chinensis	EU557091
Stenella attenuata	EU557096
Stenella coeruleoalba	EU557097
Tursiops aduncus	EU557092
Tursiops truncatus	EU557093
*Pseudorca crassidens**	HM060332
*Globicephala melas**	HM060334
*Globicephala macrorhynchus**	HM060333

Orcinus orca haplotypes*	
MtGen_1	GU187217
MtGen_2	GU187155
MtGen_3	GU187218
MtGen_4	GU187219
MtGen_5	GU187215
MtGen_6	GU187213
MtGen_7	GU187212
MtGen_8	GU187214
MtGen_9	GU187209
MtGen_10	GU187210
MtGen_11	GU187211

MtGen_12	GU187207
MtGen_13	GU187205
MtGen_14	GU187208
MtGen_15	GU187203
MtGen_16	GU187153
MtGen_17	GU187204
MtGen_18	GU187206
MtGen_19	GU187189
MtGen_20	GU187172
MtGen_21	GU187190
MtGen_22	GU187191
MtGen_23	GU187188
MtGen_24	GU187176
MtGen_25	GU187178
MtGen_26	GU187180
MtGen_27	GU187179
MtGen_28	GU187183
MtGen_29	GU187182
MtGen_30	GU187177
MtGen_31	GU187186
MtGen_32	GU187184
MtGen_33	GU187185
MtGen_34	GU187181
MtGen_35	GU187154
MtGen_36	GU187164
MtGen_38	GU187173
MtGen_39	GU187171
MtGen_40	GU187174
MtGen_41	GU187169
MtGen_42	GU187193
MtGen_43	GU187194
MtGen_44	GU187160
MtGen_45	GU187200
MtGen_46	GU187201
MtGen_47	GU187197
MtGen_48	GU187195
MtGen_49	GU187168
MtGen_51	GU187167
MtGen_52	GU187163
MtGen_53	GU187199
MtGen_54	GU187187
MtGen_55	GU187166
MtGen_56	GU187198
MtGen_57	GU187170
MtGen_58	GU187175
MtGen_59	GU187202
MtGen_60	GU187216
MtGen_61	GU187159
MtGen_62	GU187156

MtGen_63	GU187158
MtGen_64	GU187161
MtGen_65	GU187192
MtGen_66	GU187196
MtGen_67	GU187157
MtGen_50	GU187162

*source = this study

S3a: Primers used for long-range PCR amplification of cetacean mitochondrial genomes.

Primer pair	Forward primer	Reverse primer	product size
LR1	CGTGATCTGAGTTCAGACCGGAGYAATCCAGGTCG	ATTTGGAGTYGCACCAADYTTTTGG	9269
LR2	ATCCRTTGGYCTTAGGARCCAAA	TAAAAGTTTAAGTTTTATGCAATTGCCA	7456
LR3	CTTGTATGAATGGCCACACG	TAGAGGGGGTTCGATTCCTT	4858
LR4	CCTCCACCATACCACACATTC	TGTCAGTAGGGTGGAAGAGG	4966
LR2.1	GCTAATTCATGTGCTCCACACC	CGGGCTTTAACTTATCGTATGG	3999
LR2.2	CCACTGTACACACCACATACACAC	GCCACGGCTAAAAGAATGGG	3665
LR4.1	CGGATGTCCTCCACCATACCACACATTC	GGCGTATGAAGCAGATAATGAGG	2412
LR4.2	GCCCCATTTACAATCTCAGACGG	GGATAGTGGTTCAGTGTCAG	2762

*LR3 and LR4 break the LR1 product into 2 overlapping pieces
LR2.1 and LR2.2 break the LR2 product into 2 overlapping pieces
LR4.1 and LR4.2 break the LR4 product into 2 overlapping pieces

S3b: PCR conditions for long-range PCR as performed at the (a) SWFSC (using Roche Expand Long-Range PCR kit) and (b) the University of Copenhagen (using Invitrogen Platinum Taq HiFidelity). All reactions were performed in 25µl volumes using 1x buffer for the respective enzymes.

a)

Primer pair	Enzyme (units)	DMSO (%)	dNTP (µM)	Primers (each, nM)	Extension time*
LR1	0.35	8	500	600	9 min
LR2	0.35	8	500	600	7 min
LR3	0.35	0	500	600	5 min
LR4	0.35	0	500	600	5 min

*annealing time is for the first 10 "touchdown" cycles. Remaining cycles were increased by X seconds each cycle.

PCR conditions for LR1, 2, and 4

PCR cycles	Temperature	Time
Initial denaturation	92°C	2 min
Denaturation	92°C	30 sec
Annealing	65-56°C (-1°/cycle)	15 sec
Extension	68°C	(extension time)
	10 cycles	
Denaturation	92°C	30 sec
Annealing	55°C	15 sec
Extension	68°C	(extension time) +20 sec/cycle
	20-35 cycles*	
Final extension	68°C	7 min

* adjusted to obtain sufficient quantity

b)

Primer	Enzyme	MgSO4	dNTP	Primers	Extension

PCR cycles	Temperature	Time

pair	(units)	(mM)	(µM)	(each, nM)	time
LR1	0.1	2.5	200	400	9 min
LR2	0.1	2.5	200	400	7 min
LR3	0.1	2.5	200	400	5 min
LR4	0.1	2.5	200	400	5 min
LR2.1	0.1	2.5	200	400	5 min 30s
LR2.2	0.1	2.5	200	400	5 min 30s
LR4.1	0.1	2.5	200	400	5 min 30s
LR4.2	0.1	2.5	200	400	5 min 30s

re		
Initial denaturation	94°C	4 min
Denaturation	94°C	30 sec
Annealing	62°C	30 sec
Extension	68°C	(extension time)
	35 cycles	
Final extension	72°C	7 min

S4:

Variable sites (N=251) for 65 complete mitogenomes. Vertical lines represent differences from the reference sequence, haplotype CNPNRAL (MtGen19)

S5:
Prior distributions used for Bayesian analyses of divergence times, and corresponding posterior values. The Analysis I prior parameters came from lognormal distributions fit to the posterior samples from (1; McGowen pers comm.)). The *Orcinus* prior came from the Analysis I posterior on the genus. mya = million years ago. PPD = posterior probability distribution. Node numbers correspond to numbers in Figure 1a.

Phase	Node #	Taxa	Log-normal mean	Log-normal s.d.	Median (mya)	95% HPDI	Log-normal mean	Log-normal s.d.	median (mya)	95% HPDI
I	1	Cetacea	3.670	0.070	39.2	34.2 - 45.0	3.643	0.037	38.2	35.5 - 41.2
I	2	Odontoceti	3.605	0.074	36.8	31.8 - 42.5	3.628	0.037	37.6	35.0 - 40.5
I	3	Ziphiidae + Delphinida	3.528	0.079	34.1	29.2 - 39.8	3.495	0.038	32.9	30.6 - 35.5
I	4	Delphinida	3.372	0.080	29.1	24.9 - 34.1	3.326	0.041	27.9	25.7 - 30.1
I	5	Delphinoidea	3.099	0.102	22.2	18.2 - 27.1	3.063	0.053	21.4	19.2 - 23.7
I	6	Inioidea	3.045	0.132	21.0	16.2 - 27.2	2.963	0.076	19.4	16.6 - 22.3
I	7	Phocoenidae + Monodontidae	2.870	0.116	17.6	14.0 - 22.1	2.827	0.071	16.9	14.7 - 19.4
I	8	Delphinidae	2.460	0.124	11.7	9.2 - 14.9	2.556	0.071	12.9	11.2 - 14.8
I	9	Delphininae	1.476	0.143	4.4	3.3 - 5.8	1.639	0.088	5.2	4.3 - 6.1
II	10	Orcinus	-0.266	0.170	0.8	0.5 - 1.1	-0.354	0.171	0.7	0.5 - 1.0

1.   McGowen MR, Spaulding M, & Gatesy J (2009) *Molecular Phylogenetics and Evolution* **in press**.