Marine Mammal Science

MARINE MAMMAL SCIENCE, 33(1): 96–111 (January 2017)
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DOI: 10.1111/mms.12345

Genetic structure of the beaked whale genus *Berardius* in the North Pacific, with genetic evidence for a new species

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Abstract

There are two recognized species in the genus *Berardius*, Baird's and Arnoux's beaked whales. In Japan, whalers have traditionally recognized two forms of Baird's beaked whales, the common "slate-gray" form and a smaller, rare "black" form. Previous comparison of mtDNA control region sequences from three black specimens to gray specimens around Japan indicated that the two forms comprise different stocks and potentially different species. We have expanded sampling to include control region haplotypes of 178 Baird's beaked whales from across their range in the

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North Pacific. We identified five additional specimens of the black form from the Aleutian Islands and Bering Sea, for a total of eight "black" specimens. The divergence between mtDNA haplotypes of the black and gray forms of Baird's beaked whale was greater than their divergence from the congeneric Arnoux's beaked whale found in the Southern Ocean, and similar to that observed among other congeneric beaked whale species. Taken together, genetic evidence from specimens in Japan and across the North Pacific, combined with evidence of smaller adult body size, indicate presence of an unnamed species of *Berardius* in the North Pacific.

Key words: Baird's beaked whale, Arnoux's beaked whale, Ziphiidae, mitochondrial DNA, phylogenetics, population structure, cetacean.

Beaked whales are the second most speciose family of cetaceans but remain poorly understood. There are currently 22 recognized species (Committee on Taxonomy 2016), comprising 24% of all cetacean species, and nine of them have been described in the last century, including one since the millennium (Dalebout *et al.* 2002, van Helden *et al.* 2002). A few species (*e.g.*, Perrin's beaked whale, *Mesoplodon perrini*; Dalebout *et al*. 2002) are known only from remains of a few stranded animals. These diverse and unusual whales are typically found at very low density and in deep offshore or deep basin waters, and observing them is complicated by their medium-to-small size (3–13 m), deep diving behavior that keeps them below the surface for up to an hour (Mead 2009), and low surface profile that makes them difficult to spot in rougher sea-state conditions (*e.g.*, higher Beaufort level or swell) (Barlow *et al.* 2001).

The largest and one of the most common beaked whale species is Baird's beaked whale, *Berardius bairdii*, of the cold-temperate North Pacific. It is typically found along the continental slope between 1,000 and 3,000 m (though sometimes on the shelf-edge as well; Fedutin *et al.* 2015) north of 35° latitude in the western Pacific (Omura *et al.* 1955, Kasuya 1986) and north of ~24° latitude in the eastern Pacific, ranging as far north as the north Bering Sea (~62°N) (Kasuya and Ohsumi 1984, Kasuya 2009). Winter distribution is not known, and it is presumed that they move to deeper waters, with at least some time spent in the tropics, as evidenced by the presence of cookie-cutter shark bite scars (Nakano and Tabuchi 1990, Kasuya 2011, Fedutin *et al.* 2015). Its abundance has earned it the dubious honor of being one of only two beaked whale species targeted by commercial hunting historically, and it remains a species targeted by the Japanese whaling industry. Whalers have traditionally recognized a black and a slate-gray form of Baird's beaked whales around Japan (Omura *et al.* 1955). In his book on small cetaceans in the vicinity of Japan, Kasuya (2011) reviewed whaler observations that indicated the presence of at least two types of Baird's beaked whales in Japanese waters: the more common slate-gray form, and a smaller, darkly pigmented form found near the northern tip of Hokkaido in the Sea of Okhotsk. This smaller form was called by the fishermen *kuro-tsuchi* [black Baird's beaked whale] or *karasu* [crow or raven] (Kasuya 2011), and T. Yamada proposed that these represented a form distinct from Baird's and Cuvier's beaked whales (*Ziphius cavirostris*) found in the area, based on morphological examination of three stranded animals (Yamada and Tajima 2010, as reported in Kasuya 2011). Groups of the smaller whales were repeatedly observed in the Nemuro Strait off the northeast corner of Hokkaido and were characterized as being similar in body shape but only 60%–70% of the adult body size of Baird's beaked whales, and had fewer or less "intense" tooth marks on their bodies. They also exhibited scarring from cookie-cutter sharks similar to those seen regularly on Baird's beaked whales (Kasuya 2011), and indicative of at least some time spent in tropical waters. Hershkovitz (1966) reviewed all of the nominal names proposed for cetaceans and he only found that one other nominal species had been described for *Berardius* in the North Pacific. A portion of a skull from Bering Island, Commander Islands was described as B. vegae (Malm 1883). We have not examined the morphology or the genetics of this specimen, which was deposited in the Stockholm Museum of Natural History.

Genetic analysis of the two types of Baird's beaked whale around Japan was presented by Kitamura *et al.* (2013), in which they identified three specimens that had "features characteristic of the black group." These three specimens had significantly different mitochondrial control region haplotypes and differed at 1–2 nucleotide positions in the nuclear α-2-actin intron one (ACTA2I) from 64 specimens assigned to the "gray" form based on morphology and the season in which they were collected. Beaked whale species are characterized by having high interspecific control region divergence (average 8.57%; range 3.37%–20.49%) and low intraspecific diversity (0.85%; 0%–1.15%) (Dalebout *et al.* 2004, Dalebout *et al.* 2007), conforming to the "bar-coding gap" that is the basis of species identification with DNA bar codes in many well-characterized species groups (Meyer and Paulay 2005, Alfonsi *et al.* 2013). The divergence between the three black form mtDNA haplotypes identified by Kitamura *et al.* (2013) and the seven haplotypes found in the common "gray" form was 4.4%–5.1%, while the intratype distances ranged from 0.2% to 0.9% (excluding indels, calculated from sequences presented in Kitamura *et al*. 2013). Based on genetic differences and the positioning of the divergent haplotypes in a phylogenetic tree of the Ziphiidae, Kitamura *et al.* (2013) suggested that there were two "stocks" of Baird's beaked whales around Japan, and that their data supported the occurrence of cryptic species in the genus *Berardius*.

Here we present genetic analysis of 178 samples from across the North Pacific, spanning the known range (in spring/summer/fall) of Baird's beaked whales, to further characterize the genetic diversity of the genus beyond waters surrounding Japan. We identify several recently stranded and museum specimens of the "black" form based on mtDNA sequences being identical to or very similar (differing by 1–2 bp) to previously identified black form specimens (Kitamura *et al*. 2013). Addition of these specimens allows us to evaluate genetic, morphological and distributional data supporting the presence of a second species of *Berardius* in the North Pacific. Specifically, we evaluate the net DNA sequence divergence as a line of evidence supporting species-level divergence between the two forms of *Berardius* in the North Pacific, and their relationships to the other recognized congener, Arnoux's beaked whale (*Berardius arnuxii*), found only in the Southern Ocean. We also evaluate the limited evidence (suggested from traditional knowledge; Kasuya 2011) for size differences between the two forms based on external morphology of two adult black form specimens compared to published size distributions of Baird's beaked whales, and for differences in distribution or habitat use that could provide additional lines of evidence for two species of *Berardius* in the North Pacific.

Materials and Methods

Samples, DNA Extraction and Sequencing

Samples were obtained by biopsy sampling of live whales, and from stranded animals, market samples in Japan and Korea, and museum specimens (Table S1). All field-collected samples were identified as Baird's beaked whales, though one (sample 144310) was suspected to be the black form based on size and other external morphology. Two museum specimens were also identified initially as putative black form *Berardius* based on size information (see Table S1). Field-collected samples were stored either frozen at −80°C without preservative, or frozen at −20°C and preserved in 20% DMSO saturated with NaCl or in 100% ethanol. DNA was extracted from tissue samples using a silica-membrane method (DNeasy blood and tissue kit or Qiaxtractor DX reagents, Qiagen, Valencia, CA). Historical bone and tooth samples were obtained from museum and private collections and DNA was extracted in a separate "ancient DNA" laboratory as described in Morin *et al.* (2006). Market samples were extracted and DNA amplified as described in Baker *et al.* (1996).

Amplification and sequencing was performed as described in Martien *et al.* (2014) using primers H16498 (Rosel *et al.* 1994), L15829, H497 (Martien *et al*. 2014) and primer DL3c (GTGAAACCAGCAACCCGC, aka L16252, developed at SWFSC). Sequences were assembled using Sequence Scanner (v1.0, Applied Biosystems, Grand Island, NY) or Sequencher v 5.2 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI) and aligned using the MUSCLE alignment program (Edgar 2004) with default settings and eight iterations in Geneious (v6.1.6, Biomatters, Ltd., Auckland, New Zealand), and checked by eye. Unique variant sites were verified after alignment by rechecking the electropherogram alignments (confirming unique sites in both directions), and the new unique haplotype b4 was sequenced twice from separate DNA extractions. Unique 920–976 bp haplotypes have been submitted to GenBank (accession numbers KT936578–KT936586).

Resulting sequences were truncated to 431 bp (432 bp aligned) to match published sequences of *B. arnuxii* and the haplotypes of the sequences reported by Kitamura *et al.* (2013). We obtained haplotype sequences from Genbank for the seven gray form haplotypes and three black form haplotypes representing 64 sequence from Kitamura *et al.* (2013) from around Japan, and from all other recognized Ziphiidae species and from the two species of *Kogia* used as an outgroup. Accession numbers for all sequences used in this study are presented in Table S2.

Analytical Methods

Phylogenetic analysis of the Ziphiidae, including *Kogia sima* and *K. breviceps* as outgroups, was performed using Bayesian analysis in the program BEAST (v1.8; Drummond and Rambaut 2007). Sequences used for the phylogenetic analysis were as in Kitamura *et al.* (2013), except that we added four haplotype sequences of *Mesoplodon hotaula*, one additional haplotype sequence of *M. densirostris*, and the two new *Berardius* sp. haplotypes from this study (Table S2). We used the program jModelTest (v2.1; Darriba *et al.* 2012) to select the optimal mutation model (HKY) based on the Bayesian Information Criteria (BIC), with kappa and frequencies estimated by BEAST. We used the relaxed log-normal clock and a Yule speciation process. We performed 10 million Markov Chain Monte Carlo (MCMC) chains and verified convergence and tree likelihood ESS > 200 using the program Tracer (v1.5.0; Drummond and Rambaut 2007). The maximum clade credibility tree was generated using TreeAnnotator (v1.7.4) (Drummond and Rambaut 2007) with a burn-in of 1,000 trees. The input and output xml files are available from the authors on request.

A haplotype median joining network (Bandelt *et al.* 1999) was generated from the truncated 432 bp alignment of unique *Berardius sp.* haplotypes using the program PopArt (http://popart.otago.ac.nz), with epsilon set to zero. Additional mtDNA haplotypes of *B. bairdii* obtained from Japanese market sampling (Endo *et al.* 2005, 2010) were included for estimation of haplotype frequencies. Haplotype frequencies for each geographic region are in Table S3.

Analyses of net nucleotide divergence, d_A (Nei and Li 1979, Nei and Kumar 2000) was calculated using the *strataG* package (v1.0; Archer 2016) for R (R Development Core Team 2011) based on the best nucleotide model (K80) for just the *Berardius* sequences as determined with the program jModelTest (Darriba *et al.* 2012). Diagnostic sites (nucleotide positions fixed for different nucleotides between groups) were determined by visual inspection of the aligned *Berardius* haplotype sequences. Hapotype diversity and population divergence measures F_{ST}, Φ_{ST}, and χ^2 (within the gray form) were also calculated using the *StrataG* package in R (with model K80 for Φ_{ST}). Strata used for analyses are in Table S4. A Mantel test for isolation by distance (IBD) was conducted in GenAlex (v6.5; Peakall and Smouse 2006, 2012) based on pairwise Φ_{ST} genetic distances and approximate straight-line distances between geographic regions shown in Figure 1.

Results

Our samples were distributed throughout the coastal range of the species, from Japan to Mexico (Fig. 1), but few samples were taken outside of the continental shelf due to both low encounter rate and lower sampling effort (Kasuya and Ohsumi 1984, Hamilton *et al.* 2009). Haplotypes were designated as being from the black or gray form based on being identical to, or 1–2 bp different from,

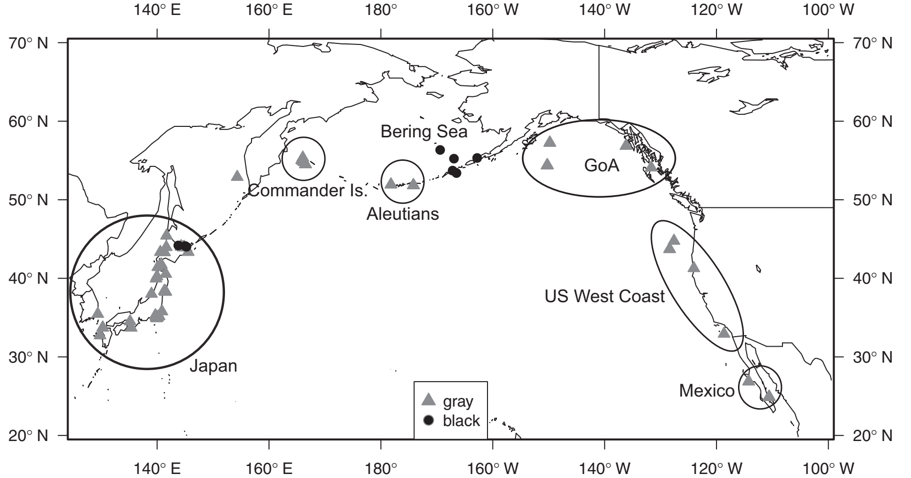

Figure 1. Distribution of samples used in this study. Sample locations around Japan were inferred from shore-based whaling stations described in Kitamura *et al.* (2013) or market samples, and do not necessarily reflect the region where the animal was collected. Some sampling locations represent multiple samples, and/or samples that yielded sequence data to identify form but not haplotype (and therefore not used in further analyses of diversity or population structure). Individual sample location details are in Table S2. Strata used for population analyses are encircled and described in Tables 3 and S4.

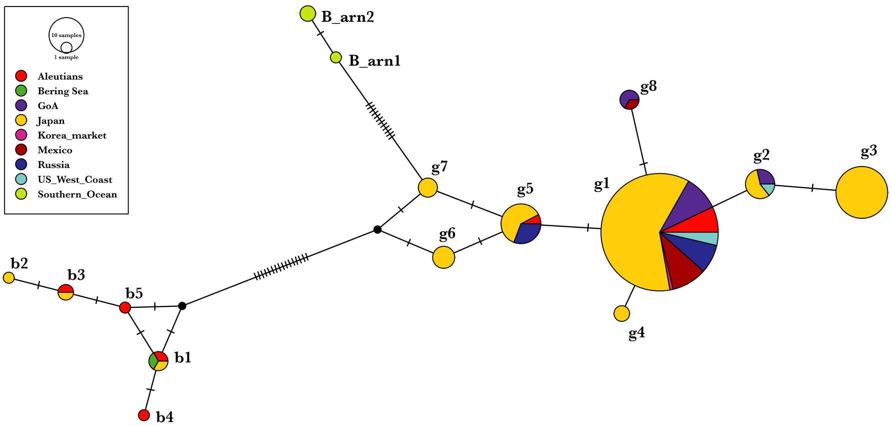

Figure 2. Haplotype network of gray and black form haplotypes. Haplotype sequences were truncated to 431 bp for alignment to published *Berardius arnuxii* sequences. Haplotypes g1–g7 and b1–b3 were described by Kitamura *et al.* (2013). Haplotypes g8, b4, and b5 are from this study. Circle sizes are proportional to sample number. Small black circles represent inferred haplotypes (not sampled). Haplotypes are colored according to geographic region as in Figure 1 for the gray form, except that "Russia" includes the Commander Islands and northern Sea of Okhotsk samples. Slash marks along the lines connecting haplotypes indicate the number of DNA sequence differences.

haplotypes associated with morphologically identified individuals from Kitamura *et al.* (2013), and being part of a monophyletic clade for each type (Fig. 2, 3). Specimens identified as the black form were clustered around northern Japan (*n* = 3; Kitamura *et al.* 2013) and in the Bering Sea and eastern Aleutians (*n* = 5; Fig. 1). One stranded specimen (SWFSC ID 144310) and two museum specimens that were identified based on external or skull morphology as likely black form were confirmed to also have mtDNA haplotypes that were identical or very similar (1–2 bp) to known black form haplotypes. Of the two additional samples identified genetically as black form, one (41749) was extensively photographed and the skeleton rearticulated for display at a high school on Unalaska Island. The other (7969) had no associated photographs or morphological data. Of the gray specimens (*n* = 169), length data of adult stranded animals were available from the Commander Islands (*n* = 1; Fedutin *et al.* 2012), northern California (*n* = 1; ID = 17152), and the Gulf of California (*n* = 10; Urban *et al.* 2007), and all were at least 9.8 m and had one of the eight gray-form haplotypes. Although length data were provided for many of Kitamura *et al.*'s (2013) specimens, maturity status was not provided except for one of the black form specimens.

We generated control region sequences from 64 *Berardius* samples, added 49 sequences from previously generated Japanese market sampling and combined the data with previously published haplotype data (*n* = 65, Dalebout *et al.* 2004, Kitamura *et al.* 2013) for a total of 178 sequenced individuals of *Berardius* (Fig. 1, Table S1). We identified two new haplotypes (b4, b5; Fig. 2) that were within 1bp of previously published haplotypes found in the black form of *Berardius*, and one new haplotype (g8) differing by 1 bp from the most common haplotype in the gray form (Fig. 2). All other sequences were identical over the 431 bp truncated sequence to previously published haplotype sequences (Kitamura *et al*. 2013). Two of the three

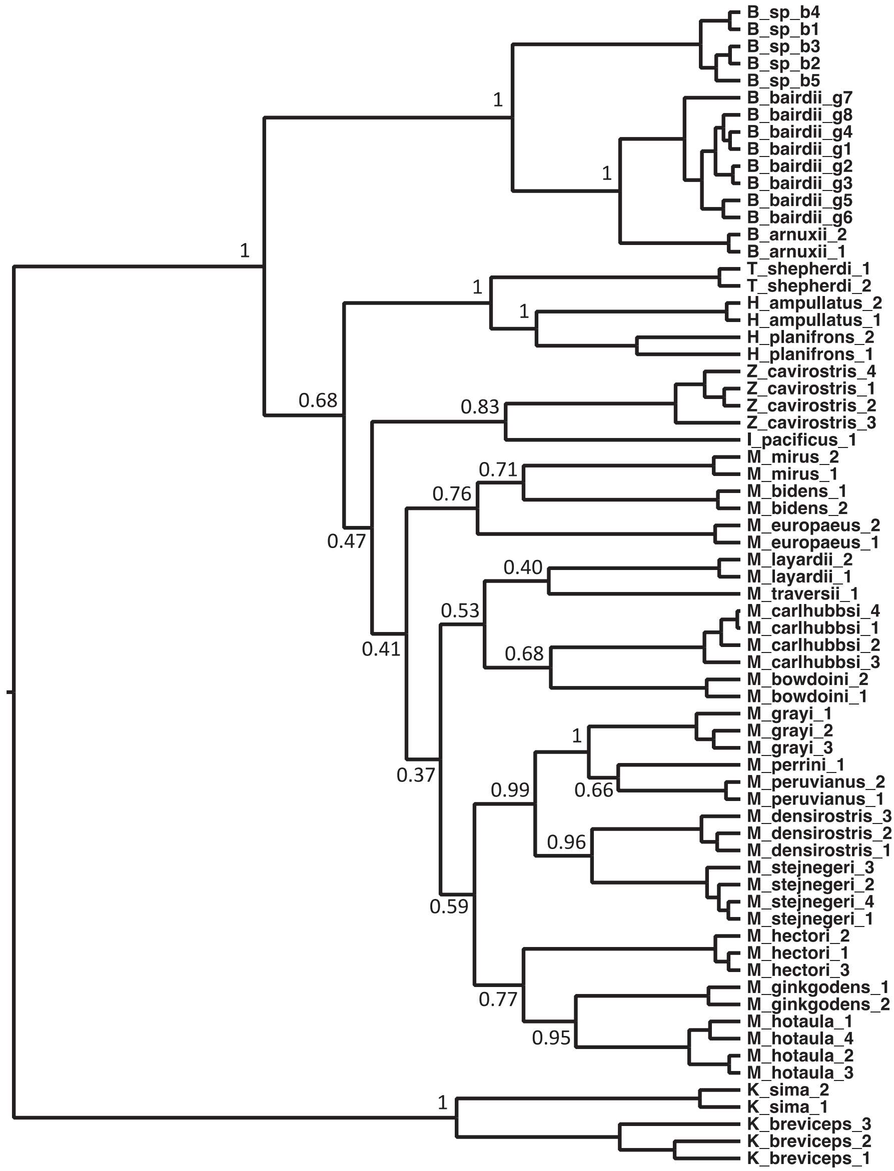

Figure 3. Phylogeny of Ziphiidae and outgroup *Kogia* samples based on mitochondrial control region sequences (481 bp alignment), including all haplotypes identified in *Berardius Bairdii* samples. Gray form haplotypes are labeled g1–8. Black form haplotypes are labeled b1–5. Numbers at nodes between species are posterior probabilities.

black form haplotypes from Japan were found in samples near the Aleutians, and three of the seven gray form haplotypes (g1, g2, g5) from Japan were found in other sampling locations (Fig. 2). The new gray form haplotype (g8) was only found in three samples from the eastern Pacific.

Phylogenetic analysis of the Ziphiidae based on the short control region sequence (481 bp when aligned to *Kogia sima* and *K. breviceps*) clustered all haplotypes of the black form of Baird's beaked whale as a monophyletic group sister to both Baird's and Arnoux's beaked whales, and the three together form a monophyletic group with high support (HPD = 1, Fig. 3). Maximum likelihood (Dalebout *et al.* 2004) and Bayesian phylogenies (this study) based on the control region have produced monophyletic species groups for most ziphiid species.

The haplotype network (Fig. 2) of *Berardius* haplotypes illustrates the relative frequencies and diversity of haplotypes across the range of the genus. Tick-marks in the figure indicate the number of nucleotide differences between haplotypes and show that diversity within each of the three types is significantly less than divergence between them. Within the gray form of Baird's whales, one haplotype (g1) was the most common throughout the North Pacific, and all but one of the haplotypes have been found in the western Pacific near Japan, with only one haplotype found just in samples from the eastern Pacific (haplotype g8). Within the black form, two of the five haplotypes were shared between Japan and the Aleutians/Bering Sea (Fig. 2).

Previous analyses of genetic differences among beaked whale species have relied on various metrics, including percent divergence and diagnostic sites (Dalebout *et al.* 2002, 2004) and net nucleotide divergence (d_A) (Dalebout *et al.* 2007, 2014; Kitamura *et al.* 2013). The net nucleotide divergence between the three *Berardius* clades ranged from 0.032 to 0.064, and there were 12–26 diagnostic sites among them (Table 1). These measures of divergence are affected by within-group diversity, so are likely to be slight overestimates due to the small number of samples available for the black form and *B. arnuxii*.

Treating the two forms of *Berardius* in the North Pacific as genetically distinct, we calculated haplotype diversity within each type and, for the gray form, in different geographic regions. Sample sizes differed widely among regions, with Japan having more than an order of magnitude more samples than any other region and including seven of the eight haplotypes found within the gray form. However, some of the strata in the eastern Pacific had similar haplotype diversity despite relatively small sample sizes (Table 2), and a unique haplotype (haplotype g8, Fig. 2). There were too few samples of the black form to look at regional differences in haplotype diversity, but overall haplotype diversity was high.

Genetic divergence among regional strata of the gray form indicates some potential population structure, especially between Japan and other strata (Table 3). However, little is known about genetic relatedness of individuals within groups, and sampling could be biased by capture or stranding of related individuals in some groups. To control for potential nonrandom sampling of related individuals, we removed all but one individual from groups of samples with the same mtDNA haplotype collected together (same date and location) and also all market samples, as they were of unknown provenance. Each successive removal of samples to control for relatedness

Table 1. Net divergence (d_A) and number of diagnostic sites (including indels) among the three *Berardius* forms.

Strata	Net divergence (d_A)	Diagnostic sites
Baird's, Arnoux's	0.032	12
Black, Baird's	0.044	16
Black, Arnoux's	0.064	26

Table 2. Summary statistics for mtDNA of the two forms of *Berardius* in the North Pacific. GoA = Gulf of Alaska. One sample from the Sea of Okhotsk (ID = 23629) had an unresolved gray-form haplotype (g1 or g4), so is not included in summary statistics).

Population	Number of samples	Number of haplotypes	Haplotype diversity
Black form (all)	8	5	0.86
Gray form			
Japan	113	7	0.57
Commander Is.	13	2	0.46
Aleutians	9	2	0.22
GoA	16	3	0.43
US West Coast	5	2	0.40
Mexico	12	2	0.17

reduced the number of significant divergence values, so that in the most conservative analysis, only one pairwise comparison among strata (Commander Island *vs*. Gulf of Alaska) showed a significant difference. In the reduced data set, at least one sample size in each pairwise comparison was <12, so conclusions from the subsampled data set about population structure of the gray form across the North Pacific are limited. A Mantel test for isolation by distance was not significant.

Discussion

Cetacean species have been described under a number of species concepts in recent years. Here, we consider a pattern of reciprocal monophyly and concordance with morphological distinctiveness (for at least some specimens used in the analysis) to be strong initial evidence of species-level distinctiveness. This satisfies the phylogenetic species concept, as interpreted by Rosenbaum *et al.* (2000) for right whales, and at least the minimum requirement of two lines of evidence required by the Lineage Concordance Species Concept, as interpreted by Dalebout *et al.* (2004), and as recommended by Reeves *et al.* (2004). We consider that additional evidences of divergence or distinctiveness from nuclear DNA loci is desirable but not necessary for our initial proposal to recognize these two forms as species. A formal description is pending a full description of a holotype for the black form and a review of the holotype for the nominate Baird's species at museums in the United States and Japan.

The topology of the Bayesian tree suggests that the Northern and Southern Hemisphere species (*B. bairdii* and *B. arnuxii*) share a common ancestor more recently than they do with the black form. Although the control region can be a poor sequence to use for phylogenetic studies, especially for inference of divergence times (Duchene *et al.* 2011), in the case of beaked whales it has been demonstrated to provide unambiguous support for species identification (Dalebout *et al.* 2004, 2007). At the genus level, the closer relationship in the phylogenetic tree between Baird's and Arnoux's beaked whales could indicate an initial species divergence between the northern and southern hemispheres, resulting in the black form in the north and the ancestor of Arnoux's and Baird's beaked whales in the south, followed by a dispersal from the Southern to the Northern Hemisphere and secondary contact between the two currently sympatric forms. Although it appears that the two forms have remained genetically isolated based on mtDNA, analysis of nuclear DNA will have to be conducted to determine whether gene flow occurred initially or is ongoing; preliminary data

Table 3. Gray-form strata divergence estimates for three pairwise estimators: F_{ST}, Φ_{ST}, and χ^2 (P-value only), with sample sizes. Pairwise comparisons were done with all samples and with samples removed to reduce the chance of nonrandom sampling of closely related individuals in some strata. The third comparison was conducted with all of the Japanese market samples removed since nothing is known about date and locations of catch. Shading indicates significance at $P < 0.05$ (dark gray) or $P \leq 0.1$ (light gray).

		All samples				Samples reduced to 1 per date/location				Samples reduced to 1 per date/location, Japan market samples removed			
Strata 1	Strata 2	n (s1/s2)	F_{ST}	Φ_{ST}	χ^2 P-value	n (s1/s2)	F_{ST}	Φ_{ST}	χ^2 P-value	n (s1/s2)	F_{ST}	Φ_{ST}	χ^2 P-value
Aleutians	GoA	9/16	0.00	0.03	0.300	3/7	−0.11	0.02	0.335	3/7	−0.11	0.02	0.361
Aleutians	Japan	9/113	0.04	0.03	0.644	3/103	−0.08	−0.04	0.558	3/54	−0.12	−0.10	0.859
Aleutians	Mexico	9/12	−0.05	0.00	0.680	3/3	−0.20	0.00	1.000	3/3	−0.20	0.00	1.000
Aleutians	Commander Is.	9/13	0.01	0.01	0.348	3/12	−0.26	−0.26	1.000	3/12	−0.26	−0.26	1.000
Aleutians	U.S. West Coast	9/5	−0.05	0.03	0.345	3/4	−0.15	0.02	1.000	3/4	−0.15	0.02	1.000
GoA	Japan	16/113	0.04	0.05	0.014	7/103	0.00	0.01	0.047	7/54	−0.02	0.00	0.201
GoA	Mexico	16/12	0.00	−0.02	0.600	7/3	−0.23	−0.17	1.000	7/3	−0.23	−0.17	1.000
GoA	Commander Is.	16/13	0.07	0.17	0.023	7/12	0.04	0.15	0.085	7/12	0.04	0.15	0.081
GoA	U.S. West Coast	16/5	−0.12	−0.09	1.000	7/4	−0.20	−0.16	1.000	7/4	−0.20	−0.16	1.000
Japan	Mexico	113/12	0.08	0.07	0.065	103/3	−0.03	0.04	0.029	54/3	−0.05	0.02	0.123
Japan	Commander Is.	113/13	0.05	0.07	0.100	103/12	0.05	0.08	0.104	54/12	0.02	0.04	0.346
Japan	U.S. West Coast	113/5	−0.01	−0.05	0.490	103/4	−0.03	−0.08	0.397	54/4	−0.06	−0.08	0.558
Mexico	Commander Is.	12/13	0.14	0.19	0.094	3/12	0.01	0.18	0.120	3/12	0.01	0.18	0.122
Mexico	U.S. West Coast	12/5	−0.01	0.06	0.537	3/4	−0.15	0.02	1.000	3/4	−0.15	0.02	1.000
Commander_Is.	U.S. West Coast	13/5	0.02	0.14	0.173	12/4	0.02	0.16	0.155	12/4	0.02	0.16	0.161

based on a single locus and samples from only around Japan indicated that the two types in the North Pacific are genetically distinct in the nuclear genome as well (Kitamura *et al.* 2013).

Sample size is heavily biased towards Japan due to sampling of commercially hunted whales, which is likely to be the reason why so many haplotypes are found in Japan compared to other locations, though it cannot be ruled out that the area around Japan harbors more genetic diversity than other areas (*e.g.*, as seen in stocks of pilot whales; Oremus *et al.* 2009, Van Cise *et al.* 2016). Most of the gray form haplotypes were shared among regions across the known range of Baird's beaked whales, but it is not clear at this point whether significant divergence among populations is a result of population structuring or IBD. Significant divergence of the Commander Islands stratum may also be due to sampling of a subpopulation that has been observed to return repeatedly to that region and may represent a local breeding stock (Fedutin *et al.* 2015). Use of nuclear markers and additional sampling to fill gaps between regions and increase sample sizes are needed to clarify population structure further.

Distribution of black form specimens was surprisingly clumped, with three samples from the Sea of Okhotsk at the northern tip of Hokkaido (Kitamura *et al.* 2013), and five samples clustered in the Bering Sea and eastern Aleutians. Although Baird's beaked whales are known to occur also in the Okhotsk and Bering Seas (Tomilin 1957; Kasuya and Ohsumi 1984; Kasuya 1986, 2011), they are relatively rare and there have been reports of a different species in the Okhotsk Sea, attributed to northern bottlenose (*Hyperoodon ampullatus*), Cuvier's (*Ziphius cavirostris*) and the black or dwarf Baird's beaked whale (Kasuya 1986, 2011). All of our Bering Sea samples of the black form were obtained north of 53° latitude (Aleutians and Bering Sea) and between 162°W and 170°W (Fig. 1), while gray form specimens were sampled to the east and west in the Aleutians, Commander Islands, and Gulf of Alaska. Sample sizes in this region remain small, but the distribution of black and gray forms suggests different distribution or habitat use.

Inference of subspecies and species based on genetic data is increasingly common but remains controversial (Hebert *et al.* 2003, Tautz *et al.* 2003, Astrin *et al.* 2006, Dupuis *et al.* 2012). For many cetaceans, where taxonomy is hindered by the dearth of skeletal materials representing the diversity within and between widely distributed species, genetic approaches may be the only way to identify new species or subspecies in the foreseeable future. At a workshop on taxonomy of cetaceans in 2004 (Reeves *et al.* 2004), participants concluded that a single "line of evidence" (*e.g.*, morphology or mtDNA sequence) was sufficient for delineating new subspecies, and two lines of evidence were needed to delineate species. Taylor *et al.*[2] have compiled a set of quantitative and qualitative guidelines to aid in consistently applying multiple lines of evidence to support subspecies and species status in cetaceans when genetic data are the primary evidence. The strength of criteria is dependent on species variables, but Taylor *et al.*[2] determined that net divergence (d_A) from mtDNA control region sequences provide a particularly good divergence metric for taxonomic delineation, with values between 0.004 and 0.02 typically found among cetacean subspecies, and values greater than 0.02 representative of species. In addition, if male-mediated gene flow cannot be ruled out (*e.g.*, only mtDNA data are available), then other evidence such as morphological differentiation or ecological or geographical separation can be used to support species status.

[2]Personal communication from Barbara Taylor, NOAA NMFS, Southwest Fisheries Science Center, 8901 La Jolla Shores Drive, La Jolla, California 92120, U.S.A., May 2016.

Based on those guidelines, we have evaluated several aspects of the mtDNA data, combined with broad geographic sampling and morphological data to determine whether the data support elevation of the black form of Baird's beaked whale to a new species within the genus *Berardius*. All of the pairwise comparisons between the three mtDNA clades in the *Berardius* phylogeny resulted in a large number of diagnostic sites and $d_A > 0.02$, strongly supporting divergence at the species level. In analyses of the control region sequences of beaked whales, Dalebout *et al.* (2002, 2004, 2007) characterized ziphiid species as generally having <2% intraspecific variation and >4% interspecific divergence. Dalebout *et al.* (2007) tested the ability to identify species based on this "barcoding gap" with a broad sampling of species within the most speciose beaked whale genus (*Mesoplodon*) and inclusion of geographically diverse samples within species, and concluded that mtDNA control region and cytochrome *b* sequences enabled "unambiguous species identifications in this group under the phylogenetic species concept." The *Berardius* clades followed this pattern as well, with <1% intraclade variation and 3.5%–6.7% divergence among the three clades. The smaller (<4%) divergence was between the two currently recognized species in different ocean basins, while the proposed new species differed from both of the recognized species by greater than 4%.

Six of the eight black form specimens were initially identified based on size and morphological differences as putative black form specimens prior to genetic ID (three from Kitamura *et al.* 2013, three from this study). Comparable morphological data in the form of external measurements of adults are only available from two of the black form specimens, but extensive external measurements from Baird's beaked whales around Japan have been previously published (Omura *et al.* 1955, Kishiro 2007). The measurements reported by Kishiro (2007) from 47 male and 31 female Baird's beaked whales from the Pacific coast of Japan most likely represent only the gray form (Kasuya 2011, Kitamura *et al.* 2013) and correspond closely to measurements routinely taken for stranding reports in the United States. The two genetically identified adult black specimens were both male and measured 733 cm (specimen z144310, Table S1) and 660 cm (specimen SNH08019; Kitamura *et al.* 2013), whereas the average adult male size from Kishiro (2007) was 998.9 cm (range 886–1,090 cm). Interestingly, although Kishiro (2007) does not recognize the possibility of two types of *Berardius* in his samples, the mean size from his Sea of Okhotsk specimens ($n = 34$) is similar, but the range is larger than in the other two regions (700–1,080 cm). Omura *et al.* (1955) also noted a bi-modal distribution of *Berardius* specimens in the Sea of Okhotsk, with several specimens of both sexes in the range of 23–25 ft (~700–760 cm). Both of these studies may reflect the inclusion of a few of the black form in the Okhotsk groups, but since the samples were not verified to be adults, it could also reflect inclusion of subadult specimens.

Based on mtDNA alone, we cannot rule out recent or ongoing male-mediated gene flow across the range, but the morphological evidence and nuclear genetic data from Japan suggest this is not the case. Evidence from nuclear DNA currently only exists for the three Japanese specimens of the black form, which all shared a single fixed difference in the α-2-actin intron one (ACTA2I) relative to 50 of the gray form from around Japan (Kitamura *et al.* 2013). Given the limited genetic signal and difficulty of obtaining DNA sequences from museum specimens, we have not attempted to expand the ACTA2I sequence data set to the range-wide *Berardius* samples.

The data presented here from multiple lines of evidence (genetics, morphology, distribution) suggest that the black form is a previously unnamed species in the genus *Berardius* that probably has a more limited range in the North Pacific or uses different habitat than Baird's beaked whale. The few specimens identified to date, despite extensive surveys of specimens around Japan and a more limited number of stranded or biopsied live animals from other regions of the North Pacific, suggest that this unnamed species is relatively rare or a less frequent visitor to continental slopes and canyons where they may be observed, caught by predominantly shore-based whalers, or drift to shore when dead. Geographic clumping of the black form in the Okhotsk and Bering Seas, while preliminary and not indicative of species-level divergence in itself, may indicate differential distributions or use of habitats by the gray and black forms. Morphological data from the black form remain scarce, and as such do not constitute a strong line of evidence for species-level difference, but the association between size, color, and/or other external morphology that is outside of the norm for Baird's beaked whales have been consistently associated with the genetically distinct group of mtDNA haplotypes, and provide further evidence by which additional specimens that may have been classified as Baird's beaked whale may be looked at more closely, especially once type specimens in Japan and the US have been characterized.

Acknowledgments

We are grateful to those who provided samples to the SWFSC Marine Mammal and Turtle Tissue Collection: Robin Baird and John Calambokidis, Cascadia Research Collective; T. Holms, Humboldt State University; Dee Allen, Kim Parsons, and Oswaldo Vasquez, NMML; Kate Wynne, University of Alaska Fairbanks Sea Grant Program; Barbara Mahoney, NMFS Alaska Regional Office and The Alaska Marine Mammal Stranding Network; Jorge Urban, Universidad Autónoma de Baja California Sur; St. George Traditional Council Island Sentinel Hertha Kashaverof; Kathy Bureck-Huntington and Dylan Peterson, Alaska Veterinary Pathology Services; Bob Pitman, John Durban, Lisa Ballance, Jay Barlow, and Karen Forney, SWFSC. Naoko Funahashi assisted with access to Japanese market samples with support from the International Fund for Animal Welfare. Tadasu Yamada suggested the specimen in the Smithsonian Marine Mammal collection for genetic identification; We thank Eric Archer, James Mead, Lee Post, Patricia Rosel, and three anonymous reviewers for helpful discussion.

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Received: 23 October 2015
Accepted: 21 May 2016

Supporting Information

The following supporting information is available for this article online at http://onlinelibrary.wiley.com/doi/10.1111/mms.12345/suppinfo.

Table S1. Sample information for samples used in this study (excluding previously published data), including the National Marine Fisheries Service (NMFS) SWFSC Marine Mammal and Marine Turtle Research (MMASTR) collection ID's (LABID), the haplotype ID's for both the short (431bp) and long (922bp) sequences, original collection ID (Field ID), collection year (Year), collection latitude and longitude and geographic region (Locality, when known), collection method (biopsy, stranding, market), tissue type, and comments on specific samples, including sex, length, and age class when known. Samples with a black-form haplotype are shaded in gray.

Table S2. Sequences used for *Berardius* and Ziphiidae analysis. New sequences generated for this study are highlighted in gray (in some cases, longer sequences of the same haplotype were generated, and submitted to GenBank with new accession numbers). Data on samples from Kitamura *et al*. 2013 are from supplementary table 1 of that publication.

Table S3. Count of haplotypes in each stratum. Haplotypes g1–g7 and b1–b3 were described by Kitamura *et al.* (2013). Haplotypes g8, b4, and b5 are from this study. Black form haplotypes (b1–b5) were not included in population analysis of Baird's beaked whales.

Table S4. Strata used for population analyses.