A DIVERGENT mtDNA LINEAGE AMONG *MESOPLODON* BEAKED WHALES: MOLECULAR EVIDENCE FOR A NEW SPECIES IN THE TROPICAL PACIFIC?

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DNA sequence data enable not only the inference of evolutionary relationships and population histories but also represent a powerful tool for uncovering hidden biodiversity, an approach that has become known as "DNA taxonomy" (Dalebout *et al.* 2002, Meegaskumbura *et al.* 2002, Hebert *et al.* 2004). The application of DNA taxonomy to beaked whales (Ziphiidae), the least known of all cetacean families, has led to some significant discoveries in recent years. These include the description of a new species from the North Pacific (*Mesoplodon perrini*; Dalebout *et al.* 2002), the

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resurrection of a long-forgotten species in the Southern Hemisphere (*M. traversii*; van Helden *et al.* 2002), and confirmation of the identity of the enigmatic "tropical bottlenose whale" (*Indopacetus pacificus*; Dalebout *et al.* 2003). The framework for these findings was provided by comprehensive data sets of DNA reference sequences for two mitochondrial (mtDNA) genes, the control region (CR) and cytochrome *b* (CYB), derived from validated voucher specimens identified by experts in beaked whale morphology. In concordance with the meticulous morphological analyses of Moore (1963, 1966, 1968) and Reyes *et al.* (1991, 1996), all beaked whale species currently recognized were found to be genetically distinct (Dalebout *et al.* 2004). These DNA data sets, together with suggested requirements for a universal DNA taxonomy, were recently published (Dalebout *et al.* 2004) and are now available through DNA-Surveillance (http://www.DNA-surveillance.auckland.ac.nz), a Web-based program for the phylogenetic identification of whales, dolphins, and porpoises (Ross *et al.* 2003).

Species identification using mtDNA sequences, also referred to as "DNA barcoding," is largely reliant on a lack of overlap between intraspecific variation and interspecific divergence (a "barcoding gap"; Meyer and Paulay 2005). For beaked whales, intraspecific mtDNA variation has been found to be low, whereas interspecif-ic divergence is comparatively high, with little to no overlap. For example, Dalebout *et al.* (2004) found that average intraspecific mtDNA variation was less than 1% (CR, 0.85%; CYB, 0.51%), whereas average interspecific divergence was greater than 8% (CR, 8.6%; CYB, 13.2%). Therefore, these two mtDNA markers appear to be well suited for species identification in this group.

Within this framework, the discovery of a highly divergent mtDNA lineage could therefore provide preliminary evidence for the existence of an unrecognized species or subspecies (Nielsen and Mikhail 2006). However, identification of such a lineage assumes that intraspecific diversity has been adequately sampled and that the barcoding gap for the group and marker in question has been accurately described (Baker *et al.* 1996, Meyer and Paulay 2005). In the reference data set of ziphiid species used by Dalebout *et al.* (2004), only two representatives per taxa were included (in some cases because only a handful of specimens of the species in question had ever been described). Given the wide geographic distribution of many beaked whales, it is possible that intraspecific variation was underestimated by these small sample sizes. Here, we test the robustness of the barcoding gap at the mtDNA CR and CYB for the most speciose beaked whale genus, *Mesoplodon,* using up to six individuals for each species. We demonstrate that the pattern of genetic differentiation observed at these markers with a smaller sample size is still valid and enables unambiguous species identifications in this group under the Phylogenetic Species Concept (PSC). Furthermore, we characterize a divergent mtDNA lineage within this genus, the taxonomic status of which will need to be determined in light of further evidence from independent nuclear markers and morphological characteristics of the specimens in question.

Interpretations of the PSC can be divided into two main classes that differ in how they define or recognize species. The "history-based" approach emphasizes the historical relatedness of species and uses genetic coalescence as a criterion for species

recognition (Baum and Donoghue 1995), whereas the "character-based" or "diagnostic" approach defines species based on the possession of fixed diagnostic characters that distinguish them from all others in a group and provide evidence that geneflow has stopped (Davis and Nixon 1992). This latter method has become widely used through the application of population aggregation analysis (PAA, Davis and Nixon 1992). For example, Rosenbaum *et al.* (2000) used diagnostic mtDNA nucleotide substitutions to provide evidence for the existence of three distinct species of right whales, *Eubalaena* spp. Although the use of PAA for species diagnosis is appealing because of its amenability to hypothesis testing, it requires *a priori* definition of population or species units and is not well suited to studies where populations are poorly sampled due to its sensitivity to sample size (Yoder *et al.* 2000). It may also be inadequate for investigations of speciose groups because the likelihood of homoplastic mutations (individuals sharing the same character state due to chance) increases with the number of taxa included in the analysis (Sanderson and Donoghue 1989).

With the "history-based" PSC approach, DNA sequences from specimens assumed to represent a given species are expected to form robust monophyletic lineages (cladistic cohesion) to the exclusion of lineages representing other described species (Milinkovitch *et al.* 2002). To date, cetacean studies using mtDNA sequences to assist in the definition of species units, identification of unknown individuals, and discovery of new species have relied largely on this criterion (*e.g.*, Baker and Palumbi 1994, Dalebout *et al.* 2002, Wada *et al.* 2003, Beasley *et al.* 2005). An extension of this approach known as the Genealogical/Lineage Concordance Species Concept (GCC; Avise and Ball 1990) has also been used explicitly by several authors (Dalebout *et al.* 2004; Caballero *et al.*, 2007). The GCC attempts to reconcile elements of the PSC with the traditional Biological Species Concept (BSC; Mayr 1963) and emphasizes the use of multiple independent genetic markers. This method was reviewed and supported by a recent specialist workshop on cetacean taxonomy (Reeves *et al.* 2004).

Assessment of patterns of within-species genetic diversity and between-species divergence in a particular group can therefore serve as rough proxy for both the historical and character-based approach and provide a useful indicator of hidden biodiversity. For example, it was the observation that there was greater genetic divergence between minke whales from the North Atlantic and Antarctic than between all other acknowledged species in the genus *Balaenoptera* that first led Arnason *et al.* (1993) to call for full species status to be conferred to the Antarctic minke whale, now known as *B. bonaerensis*. A number of recent DNA barcoding studies have also favored this approach to species discovery (*e.g.*, Hebert *et al.* 2004). However, caution should be exercised as patterns of genetic divergence, and therefore the location of the barcoding gap, cannot be generalized across species groups (Ferguson 2002), and, even where overlap between intra- and interspecific genetic diversity exists, unambiguous clades with reciprocal monophyly, pointing to the likely existence of distinct subspecies or species, can nonetheless occur (Milinkovitch *et al.* 2002).

The expanded *Mesoplodon* mtDNA database consists of seventy-one CR sequences (435 base pairs [bp]) and 65 CYB sequences (384 bp). For both markers, all 14 known species in this genus are represented by three to six sequences each (average, $n = 5$), generally representing individuals from a large portion of each species' range

(Table 1). Although it would be desirable to have sequences from the same set of individuals for both genes for each species, this was not possible due to occasional amplification problems with poor-quality DNA from decomposing stranded specimens. Sequences were aligned using the program ClustalX (Thompson *et al.* 1997) and further checked by eye using MacClade 4.07 OS X (Maddison and Maddison 1992). For the CR, the alignment required only minor adjustments for insertion–deletion (indel) mutations (10 × 1 bp indels and 1 × 2 bp indel over 435 bp). No indels were found in the CYB alignment as expected for sequences coding for a functional protein. These expanded data sets are now available on DNA-Surveillance Vs. 4.3.

In phylogenetic reconstructions using maximum likelihood and Bayesian methods, all sequences formed strongly supported species-specific clades (majority of bootstrap scores >80%, posterior probabilities >0.95), each of which was reciprocally monophyletic with respect to all other such clades (Fig. 1). Patterns of pairwise sequence divergence among *Mesoplodon* species were similar to those observed previously with a smaller sample size (Dalebout *et al.* 2004). Average intraspecific variation was low (CR, 0.6% ± 0.06%; CYB, 0.8% ± 0.09%) relative to interspecific divergence (CR, 7.4% ± 0.04%; CYB, 11.8% ± 0.04%) with little overlap (Fig. 2). For the CR, the range of intraspecific variation was 0.0%–4.3%, whereas the range of interspecific divergence was 3.4%–15.2%. For CYB, the range of intraspecific variation was 0.0%–6.4%, whereas the range of interspecific divergence was 7.0%–20.1%. Patterns of diagnostic sites among *Mesoplodon* species were also similar to those observed previously with a smaller sample size (Dalebout 2002). Thirteen of the 14 known species possessed diagnostic sites at the CR (average, 2.86 ± 0.416; range, 0–6 sites), and 13 of the 14 known species possessed diagnostic sites at the CYB (average, 2.29 ± 0.400; range, 0–5 sites). All 14 known *Mesoplodon* species possessed a minimum of two diagnostic sites over the combined CR and CYB data sets (Table 2). Note, however, that these diagnostic sites are only valid for comparisons among *Mesoplodon* species. If outgroup species are included (*e.g.*, representatives of other ziphiid genera), some homoplasies may be revealed.

The main exception to this pattern was True's beaked whale, *M. mirus*, for which genetic divergence between the North Atlantic and Southern Hemisphere forms rivaled that observed between some other *Mesoplodon* species (Fig. 2, asterisks). All *M. mirus* sequences grouped together to the exclusion of all other species (Fig. 1), but within this clade sequences from the northern (U.S. east coast) and southern (South Africa and Western Australia) forms showed a deep divergence (average CR, 4.2% ± 0.08%; CYB, 6.4% ± 0.00%) and formed strongly supported reciprocally monophyletic clades (bootstrap scores ≥90%, posterior probability ≥0.99). Furthermore, both forms possess diagnostic nucleotide substitutions that distinguish them from one another and from all other *Mesoplodon* species (CR, $n = 2$; CYB, $n = 1$). This pattern was perhaps not unexpected, however, given the disjunct allopatric distribution of *M. mirus* populations and indicates that the northern and southern forms should be considered distinct subspecies or even species (Dalebout *et al.*, unpublished data). Overall, these analyses demonstrate that even with a greatly expanded sample set and consequent increase in "noise," both the CR and CYB data sets are very well suited for the species identification of ziphiids under the Phylogenetic Species Concept.

Table 1. Geographic origins of *Mesoplodon* spp. samples used to generate mtDNA sequences for the expanded DNA Surveillance database (implemented in Vs. 4.3). Species distributions are indicated. CR-control region; CYB-cytochrome *b*; H-holotype specimen sampled.

Species	Sample origin	CR	CYB
Mesoplodon bidens, Sowerby's beaked	East Coast, USA	2	4
North Atlantic	Florida	1	—
	Scotland, UK	2	2
M. bowdoini, Andrews' beaked	New Zealand	2	3
Southern Hemisphere	South Australia	1	1
	Tasmania, Australia	1	—
	Western Australia	1	1
M. carlhubbsi, Hubbs' beaked	British Columbia	1	1
North Pacific	California	4	4
	West Coast, USA	1	—
M. densirostris, Blainville's beaked	California	1	1
Worldwide	England, UK	1	1
	French Polynesia	1	1
	New Jersey	1	1
	New Zealand	2	2
M. europaeus, Gervais' beaked	Florida	5	2
Central–North Atlantic	Colombia–Caribbean coast	1	1
	North Atlantic		2
M. ginkgodens, ginkgo-toothed beaked	Japan (H)	1	—
Indo-Pacific	California	1	—
	Taiwan	1	1
	New Zealand	2	2
M. grayi, Gray's beaked	New Zealand	4	5
Southern Hemisphere	South Australia	1	—
	Western Australia	1	—
	New South Wales, Australia	·	1
M. hectori, Hector's beaked	New Zealand	3	2
Southern Hemisphere	South Australia	1	1
	Tasmania, Australia	1	1
	Western Australia	1	1
M. layardii, straptooth	New Zealand	3	4
Southern Hemisphere	South Australia	3	—
M. mirus, True's beaked	Maryland	1	1
North Atlantic and Southern Hemisphere	New Jersey	1	1
	South Africa	1	1
	Western Australia	1	—
M. perrini, Perrin's beaked North Pacific	California (H)	5	5
M. peruvianus, lesser beaked	California	2	1
North–South Pacific	Peru	1	2
M. stejnegeri, Stejneger's beaked	Alaska	3	3
North Pacific	California	1	1
	Washington	1	1
	Japan	·	1
M. traversii, spadetoothed	New Zealand (H)	2	2
Southern Hemisphere	Juan Fernandez Islands, Chile	1	1
Total		71	65

mtDNA control region

0.005 substitutions/site
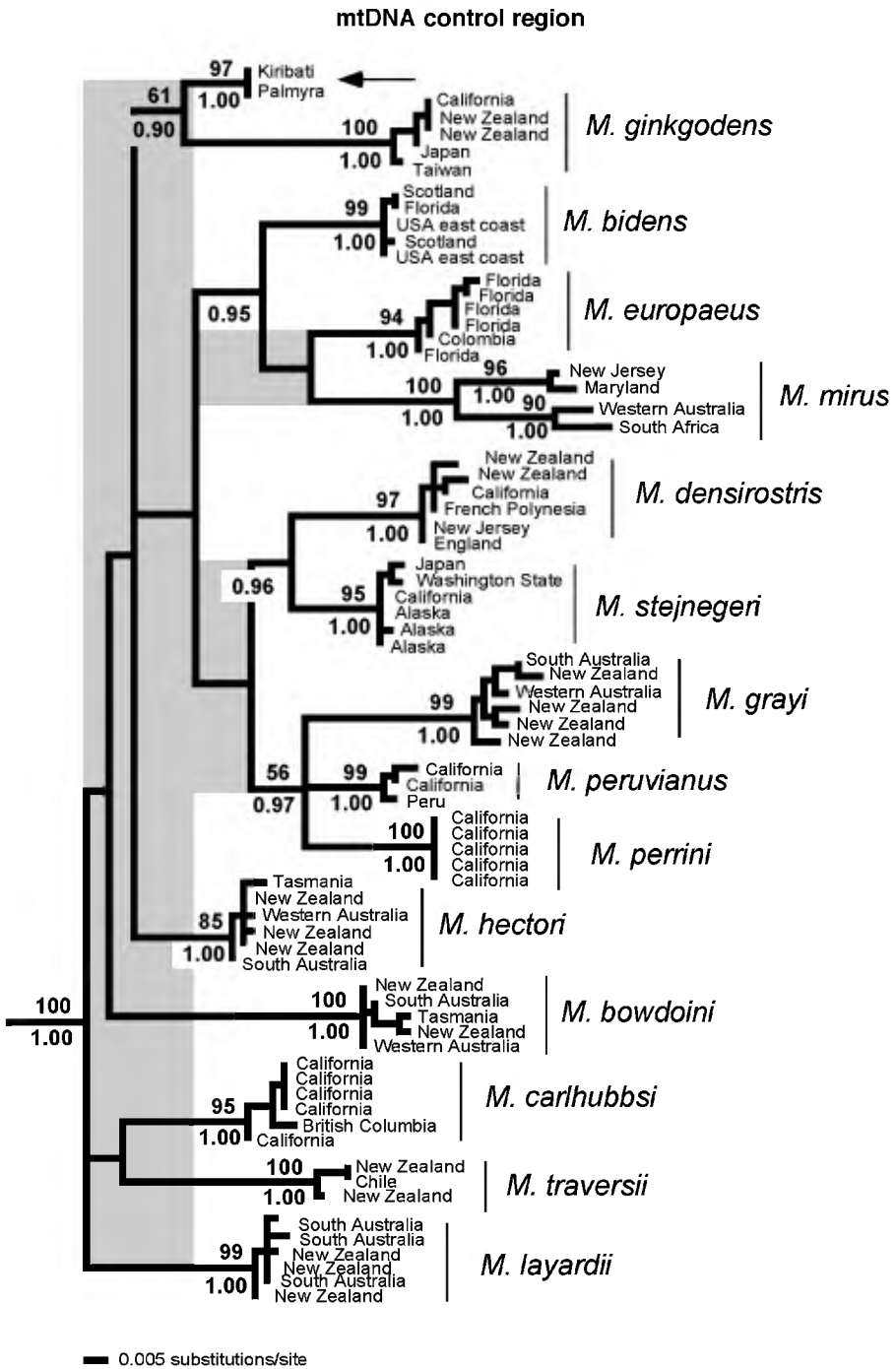
Figure 1A. Continued.

mtDNA cytochrome _b_

Figure 1B.   Continued.

←——

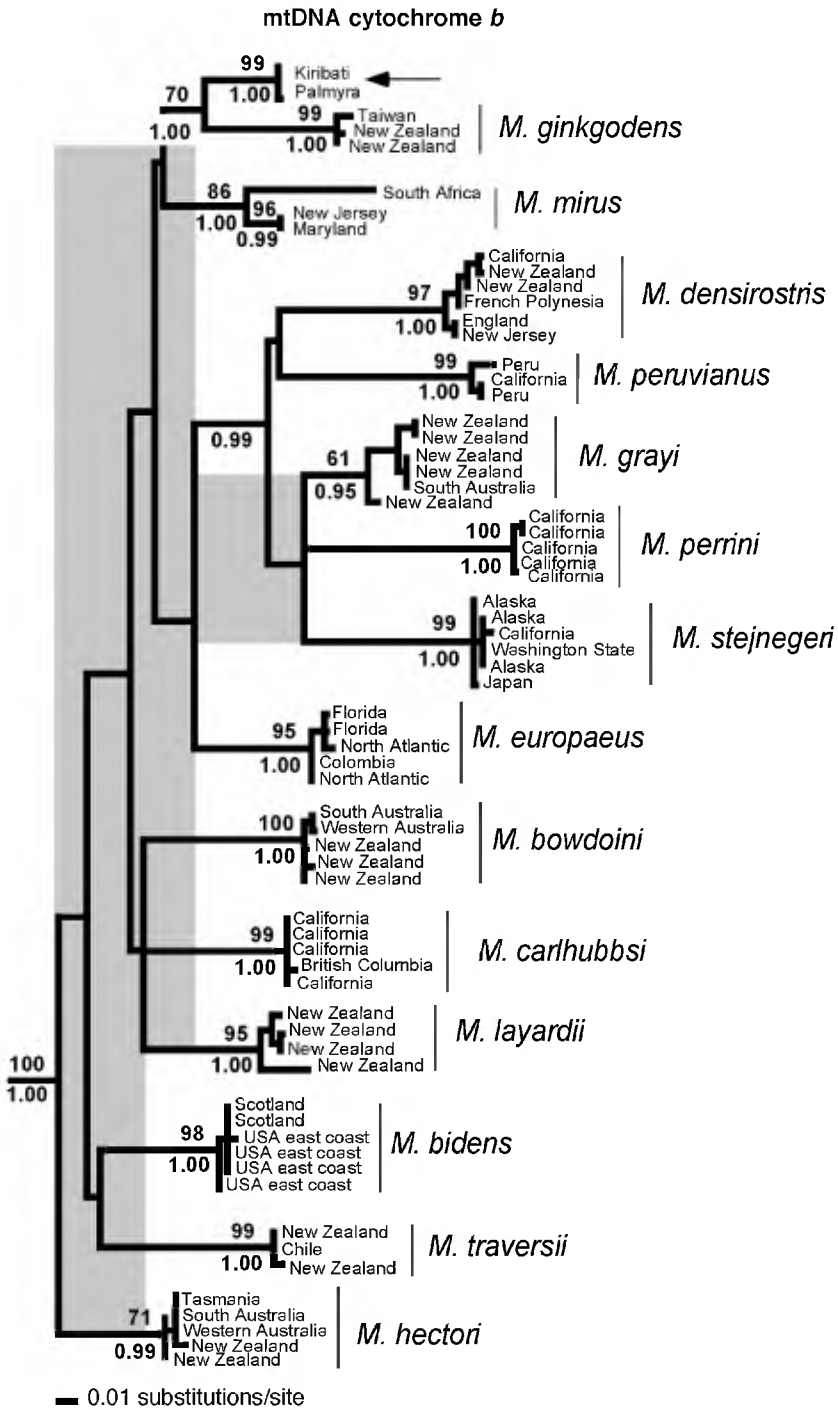*Figure 1.* Maximum likelihood (ML) trees of *Mesoplodon* beaked whale mtDNA sequences. (A) control region (CR, -ln = 2122.6503), (B) cytochrome *b* (CYB, -ln = 2368.7295). Outgroup, *Ziphius cavirostris*. Clade robustness is shown by bootstrap scores (≥60%, above branches) and Bayesian posterior probabilities (≥0.90, below branches). Note strong support of all species-specific groupings (majority of bootstrap scores >80%, posterior probabilities >0.95) and consistent patterns of low intraspecific genetic variation and high interspecific genetic divergence in this group. The arrow highlights position of the divergence lineage represented by at least three specimens (*cf. M. ginkgodens*) from the tropical Pacific. Because of the rapid rate of accumulation of mutations resulting in multiple substitutions/site (saturation), higher-level relationships between *Mesoplodon* species were generally not well resolved by these markers (gray-shaded regions, most bootstrap scores <50%). More slowly evolving nuclear markers will likely yield a more robust higher-level phylogeny for this group (Dalebout *et al.,* unpublished data). ML analyses of mtDNA sequences were performed using PAUP* 4.0b10 (Swofford 1999), with parameters estimated by Modeltest (Posada and Crandall 1998) and starting trees for heuristic searches obtained *via* neighbor joining. Full model details are available from the lead author. The robustness of nodes was assessed using 500 full heuristic nonparametric ML bootstrap replicates. Bootstrap values ≥70% were considered robust. Bayesian analyses were performed using MrBayes Vs. 3.1.2 (Huelsenbeck and Ronquist 2001) using an ML model with six substitution types and empirical base frequencies. Rate variation across sites was modeled using a gamma distribution. For CR, a proportion of sites were modeled as invariant. The Markov chain Monte Carlo search was run with four chains for 1.5–3 million generations, with trees begin sampled every 100 generations (first 1,000 trees discarded as burn in). Each BAY run was replicated to ensure a convergence of results. Posterior probabilities of ≥0.95 were considered robust.

Given the robust pattern of mtDNA variation among *Mesoplodon* beaked whales, we were surprised at the highly divergent lineage represented by several recently discovered specimens from the tropical Pacific (Fig. 1, arrows). This lineage is represented by two skulls (adult female[2] and immature of unknown sex) and associated tissue samples recovered from Palmyra Atoll Wildlife Refuge (5°52′N, 162°06′W), which appeared to be ginkgo-toothed beaked whales *M. ginkgodens* based on cranial morphology (as determined by WFP and JGM), and two tissue samples[3] collected from Tabiteuea Atoll, Republic of Kiribati (Gilbert Islands; 1°24′N, 173°6′E). These specimens formed a strongly supported clade (bootstrap score ≥99%, posterior probability 1.00), which was reciprocally monophyletic with *M. ginkgodens* (bootstrap score ≥60%, posterior probability ≥0.90) and did not group with any other *Mesoplodon* species (Fig. 1). Analyses of a combined CR-CYB data set (819 bp) using only specimens for which sequence data were held for both genes (*n* = 58) revealed the same pattern, with stronger support for reciprocal monophyly with *M. ginkgodens* (bootstrap score 91%, posterior probability 1.00; not shown). For the CR, the Palmyra and Kiribati specimens share the same haplotype and differ from *M. ginkgodens* by nineteen diagnostic sites. For the CYB, the Palmyra and Kiribati specimens differ from one another by a single nucleotide substitution and from *M. ginkgodens* by twenty-six diagnostic sites. On average, these specimens differ from *M. ginkgodens* by

[2] As determined *via* molecular sexing PCR performed by KMR.

[3] It is unclear whether these two samples (both sexed as male by DS) represent one or two animals, or if skeletal remains are available on Tabiteuea Atoll.
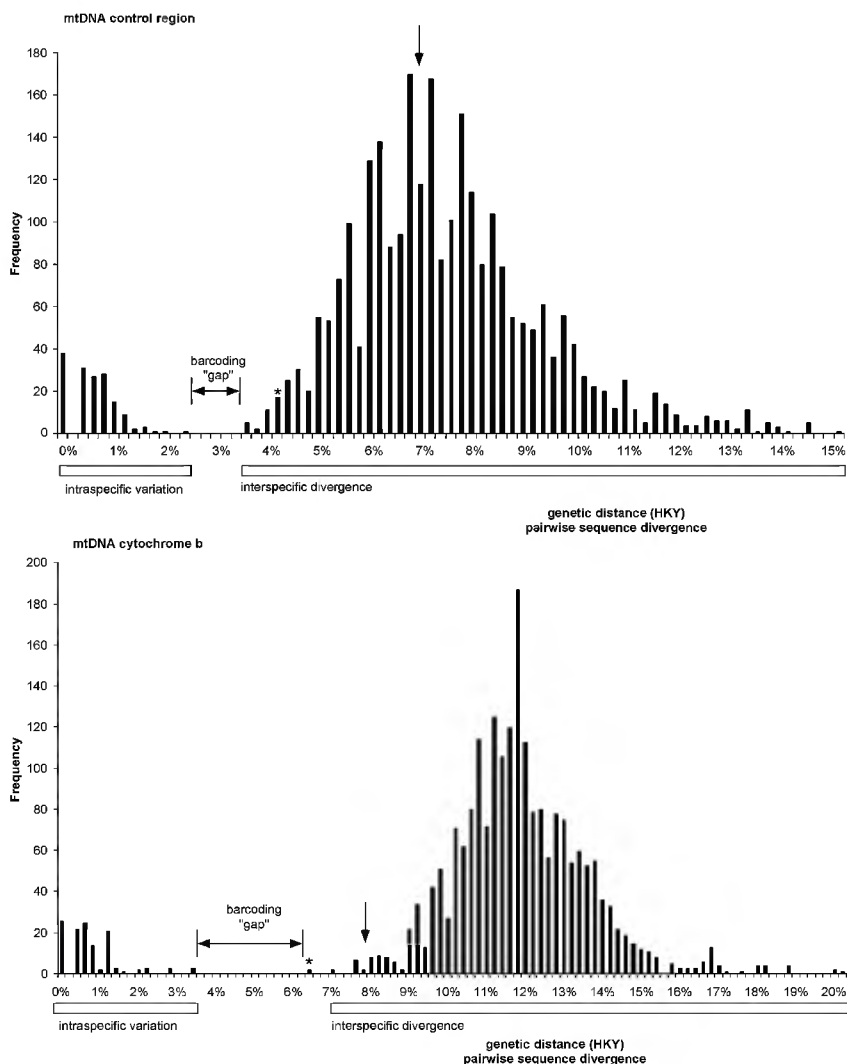
Figure 2.   Histograms of pairwise genetic distances of intraspecific diversity and inter-specific divergence for *Mesoplodon* beaked whales. Average interspecific divergence; control region, CR = $7.4\% \pm 0.04\%$; cytochrome *b*, CYB, $11.8\% \pm 0.04\%$). Asterisks highlight the comparatively high divergence observed between the northern and southern forms of *M. mirus.* (These data points were ignored for the barcoding "gap" indicated on each graph; see text for details). Arrow indicates the level of divergence observed between *M. ginkgodens* and the new lineage from the tropical Pacific.

$7.0\% \pm 0.49$ at the CR and $8.0\% \pm 0.07\%$ at the CYB. This lineage also possesses several diagnostic nucleotide substitutions distinguishing it from all other *Mesoplodon* species (CR, $n = 2$; CYB, $n = 4$).

Found in the tropical and temperate waters of the Indo-Pacific, *M. ginkgodens* was first described in 1958 from a specimen collected in Tokyo, Japan (Nishiwaki and

Table 2. Diagnostic sites (single nucleotide substitutions) distinguishing each known *Mesoplodon* species from all others in this genus at the mitochondrial DNA control region (CR) and cytochrome *b* (CYB). CR diagnostic sites include a small number of unique 1-base pair deletions. The number of diagnostic sites observed for the Kiribati/Palmyra lineage is also shown. bp, length of sequence fragment in base pairs.

| Species | Diagnostic characters | | |
	CR 435 bp	CYB 384 bp	Total
Mesoplodon bidens	3	1	4
M. bowdoini	6	5	11
M. carlhubbsi	5	2	7
M. densirostris	1	3	4
M. europaeus	3	2	5
M. ginkgodens	3	3	6
M. grayi	3	1	4
M. hectori	0	2	2
M. layardii	4	1	5
M. mirus	3	0	3
M. perrini	1	5	6
M. peruvianus	3	2	5
M. stejnegeri	1	1	2
M. traversii	4	4	8
Average	2.9	2.3	5.1
Kiribati and Palmyra specimens	2	4	6

Kamiya 1958) and is known to date from less than thirty specimens (Reeves *et al.* 2002). The five representatives sampled for this study include the holotype (Dalebout *et al.* 2004) and cover much of this species' range (Fig. 3). The divergent lineage represented by the three specimens from Kiribati and Palmyra appears to overlap in distribution with *M. ginkgodens*, albeit on an ocean-basin scale. This clearly presents a very different situation to that of *M. mirus*, the only other species in this genus for which a deep intraspecific mtDNA divergence has been observed to date. Given this pattern, we suggest that the Kiribati and Palmyra specimens could be a distinct subspecies of *M. ginkgodens* or a new species, but additional data from morphology and independent nuclear markers will be required to determine their appropriate taxonomic designation.
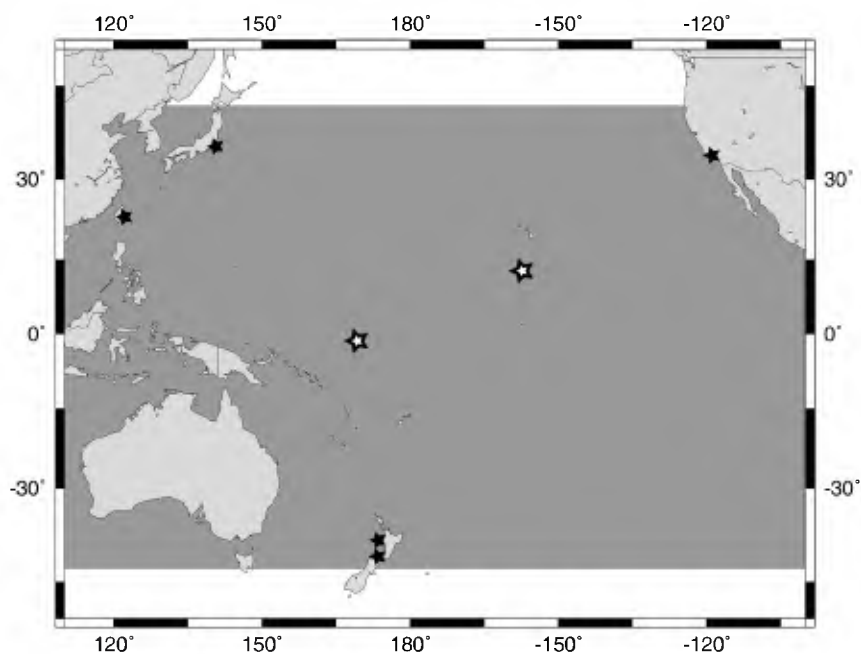
Figure 3.   Distribution of *M. ginkgodens* (gray shading) and location of confirmed specimens of this species sampled for the expanded *Mesoplodon* database (black stars). Locations of Palmyra Atoll and Kiribati, the source of specimens representing the divergent lineage, are also shown (white stars). Map created online using OMC (http://www.aquarius.geomar.de/omc).

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