Aquatic Mammals 2019, *45*(5), 525-532, DOI 10.1578/AM.45.5.2019.525

Molecular Identification of Stranded Cetaceans in Coastal China

Ting Li,[1*] Hao Wu,[2*] Caiwen Wu,[1]
Guang Yang,[1] and Bingyao Chen[1]

[1]*Jiangsu Key Laboratory for Biodiversity and Biotechnology, College of Life Sciences, Nanjing Normal University, Wenyuan Road 1, Qixia District, Nanjing 210023, China*
E-mail: bychen@njnu.edu.cn; gyang@njnu.edu.cn
[2]*Fujian Institute of Oceanography, Haishan Road 30, Xiamen, 361013, China*
**Ting Li and Hao Wu contributed equally to this manuscript*

Abstract

Drifting, stranded, or bycaught whales are often found in a decomposed state, which inhibits accurate morphological identification. Application of molecular technology is an alternative method used to identify species when decomposition is extreme. Between 2002 and 2016, six unidentified dead whales were collected in the coastal waters of China. DNA was extracted, and sequences of the mitochondrial cytochrome b (Cytb) gene or the mitochondrial control region (D-loop) were successfully amplified for BLAST (Basic Local Alignment Search Tool) alignment to construct a phylogenetic tree for species determination. As a result, two Omura's whales (*Balaenoptera omurai*), three Eden's whales (*Balaenoptera edeni edeni*), and one Blainville's beaked whale (*Mesoplodon densirostris*) were successfully identified. This is the first record of a Blainville's beaked whale being stranded in Jiangsu Province, China. Identification using these molecular techniques is providing new information on cetacean distribution and diversity in China.

Key Words: bycatch, cetacean diversity, Cytb, D-loop, whale carcasses

Introduction

Cetaceans include 89 species of whales, dolphins, and porpoises that are globally distributed in marine and riverine environments (Committee on Taxonomy, 2017; Perrin, 2018). At least 38 cetacean species occur in the waters of China (Zhou, 2004; Wang, 2011). Identification of whale carcasses can provide valuable information regarding the distribution of different species (Bijukumar et al., 2012). However, inaccurately identified animals, particularly stranded cetaceans, have limited such contributions in China (Sholl et al., 2013).

Molecular markers are useful tools for identifying the source of samples thought to be derived from threatened or endangered species (Jayasankar et al., 2007). In some cases, molecular markers are the only way to identify a cetacean when carcasses are highly degraded (Falcão et al., 2017). DNA barcoding or sequencing of mitochondrial genes, particularly cytochrome b (Cytb; Bijukumar et al., 2012; Tsai et al., 2013; Cypriano-Souza et al., 2016) and the mitochondrial control region (D-loop; Wada et al., 2003; Ottewell et al., 2016), have been used to identify cetacean species.

Six dead whales, which could not be identified morphologically, were found in the coastal waters of China between 2002 and 2016. The goal of this study was to extract genomic DNA to confirm the species identity of these stranded whales and explore if their presence supports any expanded distributions in China.

Methods

DNA Extraction

Samples from the muscle tissue of five whale carcasses and from the bone residue of one carcass were collected along coastal China (Figure 1; Table 1). DNA was extracted using a *Trelief*™ Animal Genomic DNA Kit, purchased from TSINGKE Biological Technology (Nanjing, China), and the bone sample was treated for 6 h for decalcification (Lindqvist et al., 2009). The DNA from samples of both muscle tissue and bones were then extracted by the following procedure: Under continuous concussion, muscle tissue the size of a soybean grain was digested at 56°C in 20 µl proteinase K, 200 µl high-purity water, and 200 µl Buffer gA1 for 3 h. Specific steps were performed according to the manufacturer's instructions.

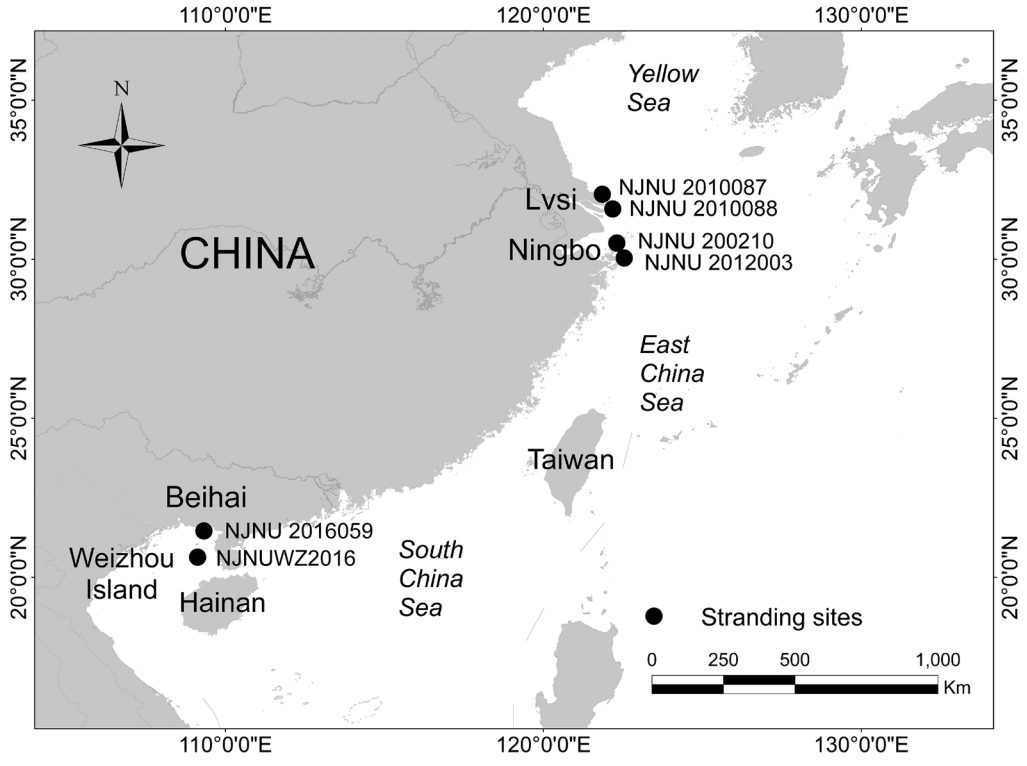

Figure 1. Map showing the four localities (black dots) from where the six cetacean species were sampled

Table 1. The relevant information on stranded whales in coastal waters of China

Number	Morphological identification	Molecular identification	Sex	Body length (cm)	Body weight (kg)	Date (d/mo/y)	Location	Remarks
NJNU200210	Unidentified	*Balaenoptera omurai*	M	703	--	4/4/2002	Ningbo, Zhejiang	Drifted
NJNU2010087	*Balaenoptera edeni* Subspecies is unknown	*B. edeni edeni*	--	1,080	--	14/102010	Lvsi, Jiangsu	Fisheries bycatch
NJNU2010088	Unidentified	*Mesoplodon densirostris*	M	395	--	15/10/2010	Lvsi, Jiangsu	Fisheries bycatch
NJNU2012003	Unidentified	*B. omurai*	--	--	--	13/3/2012	Ningbo, Zhejiang	Fisheries bycatch
NJNU2016059	*B. edeni* Subspecies is unknown	*B. edeni edeni*	F	482	1,000	2/12/2016	Beihai, Guangxi	Stranding
NJNUWZ2016	*B. edeni* Subspecies is unknown	*B. edeni edeni*	--	--	--	21/4/2018	Weizhou, Guangxi	Bone residue

Polymerase Chain Reaction (PCR)
The mitochondrial Cytb and D-loop genes were amplified by polymerase chain reaction (PCR) using the following primers: Cytb: Forward primer-5'-ATCTGCCTCTACGCTCAC-3', Reverse primer-5'-TGTCGGATGGAATACCTG-3' (Design by online tools; https://www.ncbi.nlm.nih.gov/tools/primer-blast), and D-loop: Forward primer 5'-AC ACCTCCCTAAGACTCAAGGAAG-3', Reverse primer-5'-TAGACATTTTCAGTGTCTT-GCTTT-3' (Wada et al., 2003). Primers were synthesized by TSINGKE Biological Technology. PCR was conducted in 30 µl reactions containing 14 µl ddH_2O, 0.8 µl template DNA, 0.6 µl each F/R primer, and 14 µl (2x TSINGKE) Master Mix with the following cycling profile: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s, of 54°C for 30 s, and of 72°C for 50 s; and a final extension step of 72°C for 10 min. If the sample did not amplify the first time, two amplifications were performed with the previous product as a template. All the PCR products were visualized on 1% agarose gels, and the most intense products were selected for sequencing (Bijukumar et al., 2012).

Sequence Processing
PCR products were then purified and sequenced using a commercial service (TSINGKE Biological Technology; Ottewell et al., 2016). The sequences were first edited and assembled using 'SeqMan,' implemented in *Dnastar 7.1*. Each sequence was analysed using the Basic Local Alignment Search Tool (BLAST) (Jayasankar et al., 2007; Sholl et al., 2013) available on the website of the National Center for Biotechnology Information (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi). BLAST is the most appropriate analysis tool for short input queries, for the identification of short matches, and for cross-species searches. Only the matches with an E value of 0.0 were considered because this value implies an almost zero probability of the alignment occurring by chance (Falcão et al., 2017). For the phylogenetic analyses, the sequences were aligned with the homologous sequences of different species of cetaceans downloaded in GenBank using 'MUSCLE' in *MEGA 7.0*. Phylogenetic position of the query sequences was determined using the maximum likelihood method (Ottewell et al., 2016). The best fit substitution model selected by *MEGA 7.0* was HKY+I for Cytb and HKY+G for the D-loop, and the branch support was evaluated using 1,000 bootstrap replicates.

Results

Sequences in BLAST
For the mitochondrial control region (D-loop) gene sequence, samples NJNU200210, NJNU2012003, NJNU2010087, NJNU2016059, and NJNU2010088 yielded 904 bp, 903 bp, 903 bp, 913 bp, and 804 bp of clean DNA sequence, respectively. The bone residue of NJNUWZ2016 could not be successfully sequenced.

Samples NJNU200210 and NJNU2012003 showed 99% identity to the sequences of Omura's whale (*Balaenoptera omurai*) in GenBank (Supplemental Material 1; supplementary material for this article can be found in the "Supplementary Material" section of the *Aquatic Mammals* website: https://www.aquaticmammalsjournal.org/index.php?option=com_content&view=article&id=10&Itemid=147). Similarly, the sequences of samples NJNU2010087 and NJNU2016059 showed 99 to 100% identity to Eden's whale (*Balaenoptera edeni edeni*; Supplemental Material 2). Sample NJNU2010088 showed 99% sequence identity to Blainville's beaked whale (*Mesoplodon densirostris*; Supplemental Material 3).

Except for sample NJNU2010088, sequences of the mtDNA Cytb gene, with effective lengths of 480 bp, 1,130 bp, 1,015 bp, 358 bp, and 346 bp, were successfully obtained for samples NJNU200210, NJNU2012003, NJNU2010087, NJNU2016059, and NJNUWZ2016, respectively. Samples NJNU200210

Table 2. GenBank accession numbers of the D-loop and Cytb sequences of stranded whales in this study

	GenBank accession number	
Sample number	D-loop	Cytb
NJNU200210	MH844102	MH844107
NJNU2012003	MH844103	MH844108
NJNU2010087	MH844104	MH844111
NJNU2016059	MH844105	MH844109
NJNU2010088	MH844106	--
NJNUWZ2016	--	MH844110

and NJNU2012003 were found to have the highest similarity with the Omura's whale, reaching 99 to 100% (Supplemental Material 4). Likewise, samples NJNU2010087, NJNU2016059, and NJNUWZ2016 showed 100% identity to Eden's whale (Supplemental Material 5). The GenBank accession numbers of the D-loop and Cytb sequence data generated in this study are given in Table 2.

Phylogenetic Analysis

Thirty-three D-loop sequences and 19 Cytb sequences, respectively, were used for the phylogenetic analysis. Both baleen and toothed whales can be identified by the D-loop sequence, so the Zebu (*Bos indicus*), a proximal species, was chosen as the outgroup (Figure 2). For Cytb, the killer whale (*Orcinus orca*) was included as an outgroup because only baleen species sequences were contained. Two phylogenetic trees for Cytb sequences were constructed for NJNU200210 and NJNU2012003 (Figure 3) and for NJNU2010087, NJNU2016059, and NJNUWZ2016 (Figure 4) because the sequence positions of these samples were slightly different.

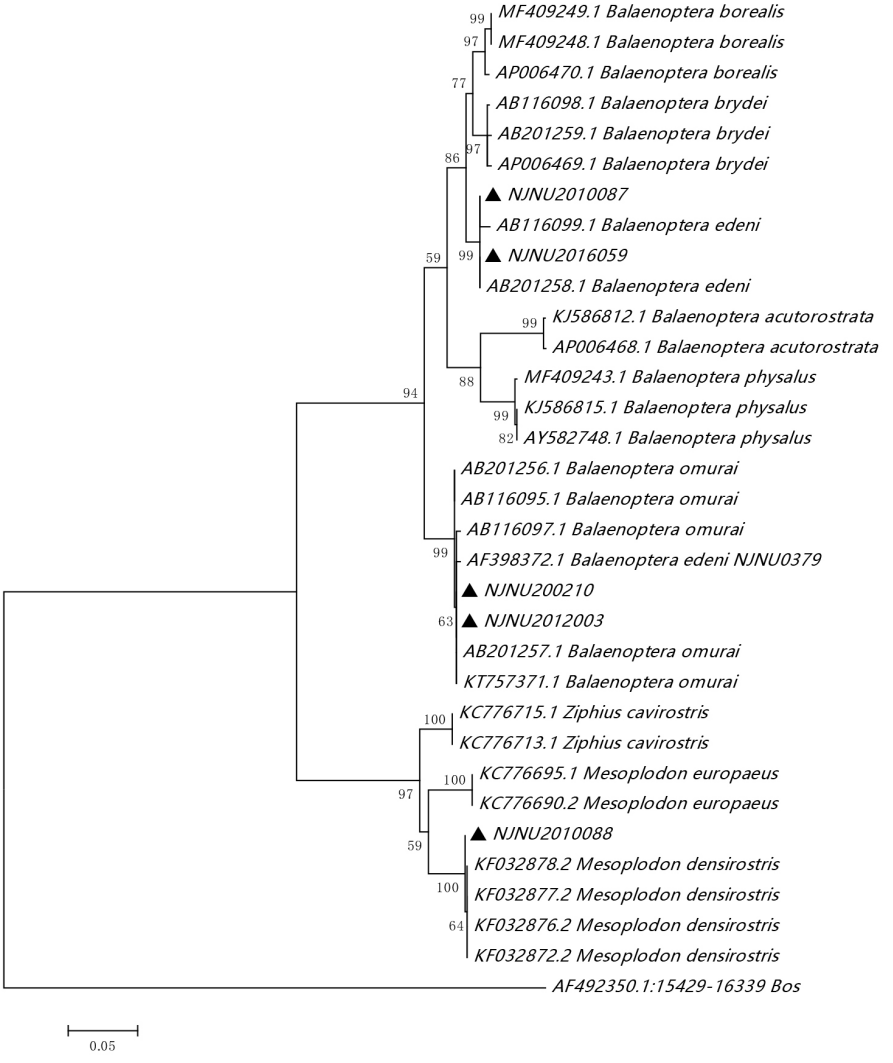

Figure 2. Clades of conspecific sequences for the five studied cetacean species showing a match between this present study and GenBank sequences. Maximum likelihood tree based on D-loop partial sequences; the numbers on the tree branches indicate bootstrap values.

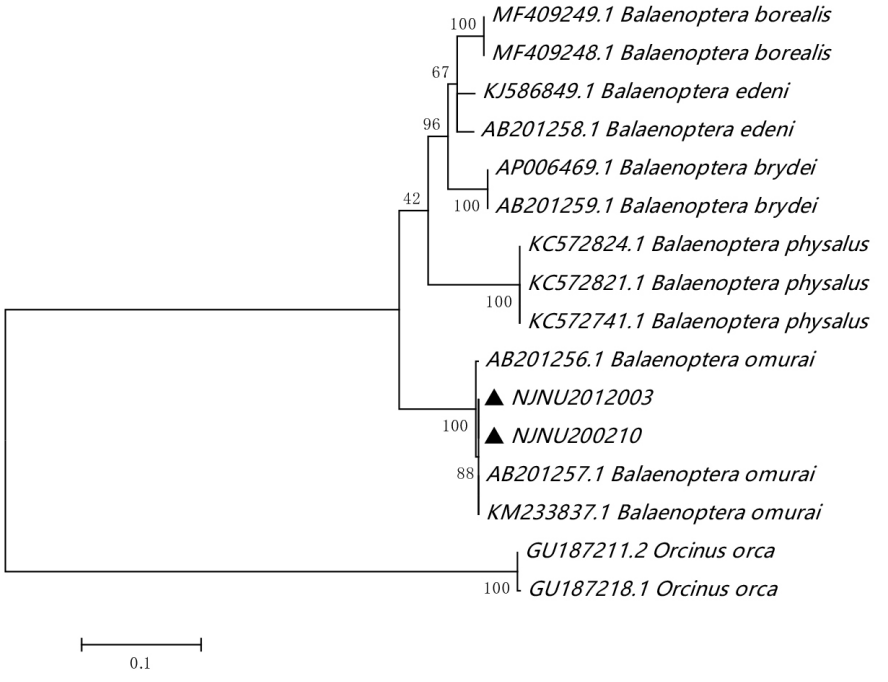

Figure 3. Maximum likelihood phylogram using Cytb partial sequences of the NJNU200210 and NJNU2012003 samples compared with other reference sequences of *Balaenoptera* spp. in GenBank. The numbers on the tree branches indicate bootstrap values.

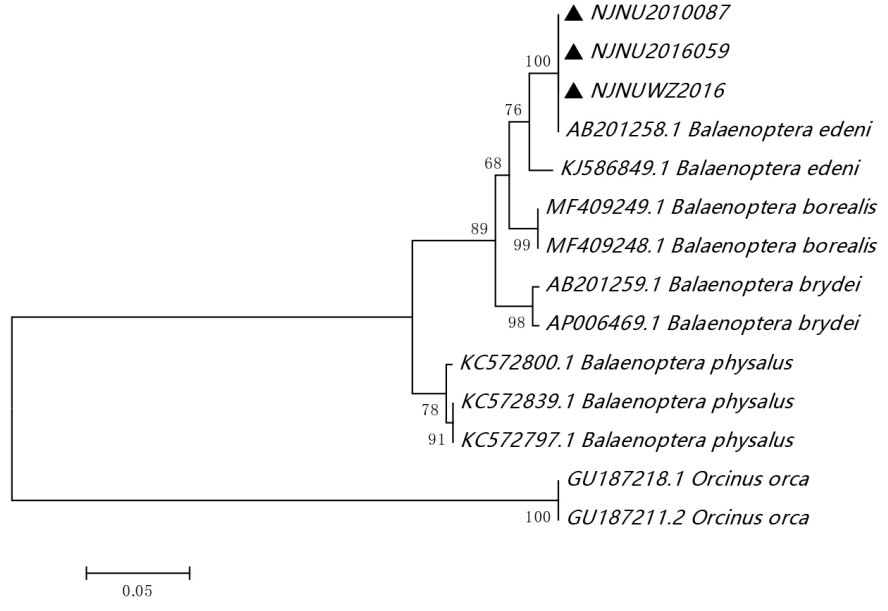

Figure 4. Maximum likelihood phylogram using Cytb partial sequences of the NJNU2010087, NJNU2016059, and NJNUBHWZ2016 samples compared with other reference sequences of *Balaenoptera* spp. in GenBank. The numbers on the tree branches indicate bootstrap values.

Phylogenetic trees using D-loop and Cytb sequences (Figures 2, 3 & 4) were similar, and the NJNU200210 and NJNU2012003 samples clustered with the *B. omurai* sequences in GenBank (Figures 2 & 3). The NJNU2010087, NJNU2016059, and NJNUWZ2016 samples were clustered with *B. e. edeni* (Figures 2 & 4). The NJNU2010088 sample clustered with other Blainville's beaked whales (Figure 2).

Discussion

Omura's whale is a rare species known from a limited number of specimens and sightings (Ottewell et al., 2016). It was formally described in 2003 (Wada et al., 2003; Ottewell et al., 2016). Before 2003, Omura's whale and Bryde's whale (*B. e. edeni*/*brydei*) were described in the "Bryde's whale complex" (George et al., 2011; Bijukumar et al., 2012; Cerchio et al., 2015; Ottewell et al., 2016). Therefore, whales previously described in this complex may have been misidentified. For example, in 2002, a sequence submitted to GenBank (GenBank accession number AF398372.1) was misidentified as an Eden's whale (Yang et al., 2002) when, in fact, it was likely an Omura's whale (Figure 2; Supplemental Material 1).

The global distribution of Omura's whale remains unclear. The known distribution of Omura's whale is primarily in the tropical/subtropical Indian and west Pacific Oceans but may extend to all ocean basins (Yamada, 2009). In China, the Omura's whale is found around Taiwan Province (Wang, 2011), Zhejiang Province, Fujian Province, and Guangdong Province (Gao, 2004; Zhu et al., 2004; Wang, 2011). In the present study, the sample found in Ningbo, Zhejiang Province, was also identified as Omura's whale, providing support for a potential expansion of the distribution of the Omura's whales into Zhejiang Province.

Bryde's whales are found mainly in the warm waters of the Pacific, Atlantic, and Indian Oceans (Visser et al., 2016). Along the coast of the Chinese mainland, Zhejiang, Jiangsu, Shanghai, Fujian, and Guangdong Provinces all have recorded sightings of Bryde's whales (Wang, 2011). However, the taxonomy of Bryde's whales has not been resolved. Wada et al. (2003) divided Bryde's whale into two distinct species—*B. brydei* and *B. edeni*—while Kershaw et al. (2013) assigned these species a subspecific status—*B. e. edeni* and *B. e. brydei*, respectively. Sample NJNU2010087, from Lvsi, Jiangsu Province, was identified as Eden's whale, which potentially extends their known distribution. The present study appeared to support the taxonomic classification into two distinct species, but further studies are needed (Figures 2, 3 & 4).

Beaked whales (family Ziphiidae) account for 25% of all cetacean species diversity, second only to the dolphin family (Pitman, 2002; MacLeod et al., 2006). Nevertheless, beaked whales are one of the least known groups of large mammals. Blainville's beaked whales are mainly distributed in tropical and subtropical waters, and some stranded whales have been found along the mainland coast of China (Jefferson et al., 1993; Wang et al., 2009), for example, in Shandong, Liaoning, Fujian, and Zhejiang Provinces and Shanghai (Wang, 2011). To our knowledge, this is the first report of a Blainville's beaked whale in Jiangsu Province.

The causes for whale strandings are poorly understood, but many potential explanations exist—for example, natural causes such as weakness due to old age or infection (Weisburd, 1984) or foraging too close to the coast (Reynolds & Odell, 1991). Alternatively, anthropogenic factors, such as ship collisions (Chen et al., 2016; Rockwood et al., 2017), naval exercises (Geraci & Lounsbury, 2005; Arbelo et al., 2013), marine pollution (Fire et al., 2010; Letcher et al., 2010), and fisheries bycatch (Schipper et al., 2008) have also been suggested as causes of cetacean strandings.

To summarize, in the present study, we demonstrated the accuracy and rationality of DNA molecular techniques for identifying stranded cetaceans. Also, the location of cetacean strandings reveals a potential range expansion, thereby improving our knowledge about the abundance and distribution of cetaceans in China.

Acknowledgments

The present study was financially supported by the National Key Programme of Research and Development, Ministry of Science and Technology (2016YFC0503200); the National Natural Science Foundation of China (31630071); the NSF of Jiangsu Province of China (BK20171475); the Ministry of Agriculture of the People's Republic of China "Chinese White Dolphin Action Plan 2017-2026"; Ocean Park Conservation Foundation Hong Kong (OPCFHK; MM04-1819); and the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

Literature Cited

Arbelo, M., Los Monteros, A. E., Herrã, E. P., Andrada, M., Sierra, E., Rodríguez, F., . . . Fernández, A. (2013). Pathology and causes of death of stranded cetaceans in the Canary Islands (1999-2005). *Diseases of Aquatic Organisms*, *103*(2), 87-99. https://doi.org/10.3354/dao02558

Bijukumar, A., Jijith, S. S., Kumar, U. S., & George, S. (2012). DNA barcoding of the Bryde's whale *Balaenoptera edeni* Anderson (Cetacea: Balaenopteridae) washed ashore along Kerala coast, India. *Journal of Threatened Taxa*, *4*(3), 2436-2443. https://doi.org/10.11609/JoTT.o2859.2436-43

Cerchio, S., Andrianantenaina, B., Lindsay, A., Rekdahl, M., Andrianarivelo, N., & Rasoloarijao, T. (2015). Omura's whales (*Balaenoptera omurai*) off northwest Madagascar: Ecology, behaviour and conservation needs. *Royal Society Open Science*, *2*(10), 150301. https://doi.org/10.1098/rsos.150301

Chen, B., Xu, X., Jefferson, T. A., Olson, P. A., Qin, Q., Zang, H., . . . Yang, G. (2016). Conservation status of the Indo-Pacific humpback dolphin (*Sousa chinensis*) in the northern Beibu Gulf, China. *Advances in Marine Biology*, *73*, 119-139. https://doi.org/10.1016/bs.amb.2015.10.001

Committee on Taxonomy. (2017). *List of marine mammal species and subspecies*. Lawrence, KS: Society for Marine Mammalogy. Retrieved from www.marinemammalscience.org

Cypriano-Souza, A. L., Meirelles, A. C. O., & Carvalho, V. L. (2016). Rare or cryptic? The first report of an Omura's whale (*Balaenoptera omurai*) in the South Atlantic Ocean. *Marine Mammal Science*, *33*(1), 80-95. https://doi.org/10.1111/mms.12348

Falcão, L. H. O., Campos, A. S., Freitas, J. E. P., Furtado-Neto, M. A. A., & Faria, V. V. (2017). Molecular identification of cetaceans from the west Atlantic using the E3-I5 region of COI. *Genetics & Molecular Research*, *16*(2), gmr16029607. https://doi.org/10.4238/gmr16029607

Fire, S. E., Wang, Z., Berman, M., Langlois, G. W., Morton, S. L., Sekula-Wood, E., & Benitez-Nelson, C. (2010). Trophic transfer of the harmful algal toxin domoic acid as a cause of death in a minke whale (*Balaenoptera acutorostrata*) stranding in southern California. *Aquatic Mammals*, *36*(4), 342-350. https://doi.org/10.1578/AM.36.4.2010.342

George, S., Meenakshi, K., & Bijukumar, A. (2011). Molecular taxonomy of marine mammals stranded along Kerala coast, India. *Current Science*, *100*(1), 117-120.

Geraci, R., & Lounsbury, V. J. (Eds.). (2005). *Marine mammals ashore: A field guide for stranding* (2nd ed.). Baltimore, MD: National Aquarium and Baltimore Press.

Jayasankar, P., Anoop, B., Peter, R., Afsal, V. V., & Rajagopalan, M. (2007). Species of a whale and an unknown fish sample identified using molecular taxonomy. *Indian Journal of Fisheries*, *54*(3), 339-343.

Jefferson, T. A., Leatherwood, S., & Webber, M. A. (1993). *Marine mammals of the world*. Rome: Food and Agriculture Organization of the United Nations.

Kershaw, F., Leslie, M. S., Collins, T., Mansur, R. M., Smith, B. D., Minton, G., . . . Rosenbaum, H. C. (2013). Population differentiation of 2 forms of Bryde's whales in the Indian and Pacific Oceans. *Journal of Heredity*, *104*(6), 755-764. https://doi.org/10.1093/jhered/est057

Letcher, R. J., Bustnes, J. O., Dietz, R., Jenssen, B. M., Jørgensen, E. H., Sonne, C., . . . Gabrielsen, G. W. (2010). Exposure and effects assessment of persistent organohalogen contaminants in arctic wildlife and fish. *Science of the Total Environment*, *408*(15), 2995-3043. https://doi.org/10.1016/j.scitotenv.2009.10.038

Lindqvist, C., Probst, A., Martin, A. R., Øystein, W., & Bachmann, L. (2009). Molecular species identification of historical whale remains from South Georgia. *Marine Mammal Science*, *25*(1), 229-238. https://doi.org/10.1111/j.1748-7692.2008.00248.x

MacLeod, C. D., Perrin, W. F., Pitman, R., Barlow, J., Balance, L., D'Amico, A., . . . Waring, G. T. (2006). Known and inferred distributions of beaked whale species (Cetacea: Ziphiidae). *Journal of Cetacean Research and Management*, *7*(3), 271-286.

Ottewell, K., Coughran, D., Gall, M., Irvine, L., & Byrne, M. (2016). A recent stranding of Omura's whale (*Balaenoptera omurai*) in western Australia. *Aquatic Mammals*, *42*(2), 193-197. https://doi.org/10.1578/AM.42.2.2016.193

Perrin, W. F. (2018). *World Cetacea database*. Retrieved from www.marinespecies.org/cetacea

Pitman, R. L. (2002). Indo-Pacific beaked whale, *Indopacetus pacificus*. In W. F. Perrin & B. Würsig (Eds.), *Encyclopedia of marine mammals* (pp. 615-617). San Diego, CA: Academic Press.

Reynolds III, J. E., & Odell, D. K. (1991). *Marine mammal strandings in the United States*. *Proceedings of the Second Marine Mammal Stranding Workshop* (NOAA Technical Report NMFS 98). Washington, DC: National Oceanic and Atmospheric Administration. 166 pp.

Rockwood, R. C., Calambokidis, J., & Jahncke, J. (2017). High mortality of blue, humpback and fin whales from modeling of vessel collisions on the U.S. west coast suggests population impacts and insufficient protection. *PLOS ONE*, *12*(8), e0183052. https://doi.org/10.1371/journal.pone.0183052

Schipper, J., Chanson, J. S., Chiozza, F., Cox, N. A., Hoffmann, M., Katariya, V., . . . Young, B. E. (2008). The status of the world's land and marine mammals: Diversity, threat, and knowledge. *Science*, *322*, 225-230. https://doi.org/10.1126/science.1165115

Sholl, T. G. C., Moura, J. F. D., Ott, P. H., Bonvicino, C. R., Reis, E. C., Tavares, D. C., & Siciliano, S. (2013). Cytochrome b sequencing for the species identification of whale carcasses washed ashore in Brazil. *Marine Biodiversity Records*, *6*, e30. https://doi.org/10.1017/S1755267212001157

Tsai, L. C., Lee, J. C., Shen, L. L., & Hsieh, H. M. (2013). Identification of the seized meats products for suspected Cetacea. *Forensic Science Journal*, *12*, 9-14.

Visser, F., Curé, C., Kvadsheim, P. H., Lam, F-P. A., Tyack, P. L., & Miller, P. J. O. (2016). Disturbance-specific social responses in long-finned pilot whales, *Globicephala melas*. *Scientific Reports*, *6*, 28641. https://doi.org/10.1038/srep28641

Wada, S., Oishi, M., & Yamada, T. K. (2003). A newly discovered species of living baleen whale. *Nature*, *426*, 278-281. https://doi.org/10.1038/nature02103

Wang, P. L. (Ed.). (2011). *Chinese cetaceans*. Beijing: Chemical Industry Press.

Wang, P. L., Ma, Z. Q., Gao, W. B., & Wang, Z. H. (2009). Beaked whales in coastal waters of China. *Chinese Journal of Wildlife*, *30*(2), 59-63.

Weisburd, S. (1984). Whales and dolphin use magnetic "roads." *Science News*, *126*, 391. https://doi.org/10.2307/3969072

Yamada, T. K. (2009). Omura's whale: *Balaenoptera omurai*. In W. F. Perrin., B. Würsig, & J. G. M. Thewissen (Eds.), *Encyclopedia of marine mammals* (2nd ed., pp. 799-801). London: Academic Press. https://doi.org/10.1016/B978-0-12-373553-9.00187-5

Yang, G., Liu, H., Zhou, K., & Ji, G. (2002). Identification of a *Balaenoptera edeni* specimen by using mitochondrial DNA sequences. *Chinese Journal of Zoology*, *37*(4), 35-38.

Zhou, K. (Ed.). (2004). *Fauna Sinica, Mammalia: Vol. 9. Cetacea, Carnivora: Phocoidea, Sirenia*. Beijing: Science Press.

Zhu, Q., Yamada, T. K., & Wang, P. L. (2004). Biodiversity and conservation of cetaceans in China. *National Science Museum Monographs*, *24*, 287-292.