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Cytochrome *b* sequencing for the species identification of whale carcasses washed ashore in Brazil

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Carcasses of whales provide much valuable information on their natural history. However, some specimens cannot be identified in the field due to the advanced state of decomposition. In this study, the DNA was extracted and the mitochondrial cytochrome b *gene was amplified by polymerase chain reaction and sequenced for four carcasses of possible mysticeti (GEMM: 075, 088, 135 and GEMARS: 1302). A blast search using the nucleotide–nucleotide basic local alignment (blastn) search tool was conducted using the generated sequences. Samples GEMM 075 and GEMARS 1302 showed 98% identity to one sequence of* Balaenoptera acutorostrata. *Samples GEMM 088 and GEMM 135 showed 99% identity to sequences from* Balaenoptera edeni *and* Megaptera novaeangliae*, respectively. A neighbour-joining tree was generated using sequences from GenBank from all species of balaenopterid that occur on the coast of Brazil. The results showed that all carcasses analysed were correspondent to species from the family Balaenopteridae already recorded in Brazil.*

Keywords: whales, Brazil, carcass, strandings, cytochrome *b*

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INTRODUCTION

In Brazil, baleen whales are represented by eight species distributed in two families: Balaenidae and Balaenopteridae. They occur in Brazilian waters mainly during winter and spring when they are moving to their reproductive areas in warm waters (Zerbini *et al.*, 2004). From the eight extant species, two are considered data deficient, three are endangered, and the others are considered as of least concern by the IUCN (IUCN, 2012). Whales have been found beached throughout human history and different reasons for this phenomenon have been proposed (Geraci & Lounsbury, 2005). The reasons whales strand are not yet fully understood. While some single strandings may be accounted for by a whale dying at sea and being washed ashore, many strandings are believed to occur due to other factors (Walsh *et al.*, 1990). There could be many natural reasons like rough weather, weakness due to old age or infection, difficulty giving birth, hunting too close to shore and navigational mistakes (Weisburd, 1984; Reynolds & Odell, 1991). Human-related causes, such as military sonar use, vessel collision, by-catch and marine pollution, have also been implicated in some cetacean strandings (Geraci Lounsbury, 2005).

Carcasses provide much valuable information on the natural history of a species. However, many carcasses are washed ashore in advanced decomposition preventing further investigations, principally in tropical regions. On several occasions, even positive identification is not possible due to the advanced decomposition of the carcasses. In these cases, the identification can be made using the sequencing of the mitochondrial DNA (Kocher *et al.*, 1989; Carr *et al.*, 2002). Such source of DNA is present in high copy number in vertebrate cells; and any particular mitochondrial gene is far more likely to survive post-mortem degradation, enzymatic breakdown, and mechanical damage than is any typical nuclear sequence (Hermann & Hummel, 1994).

In this study, we report the molecular identification of four carcasses washed ashore in Rio de Janeiro (south-eastern

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Brazil) and Rio Grande do Sul State (Southern Brazil). The carcasses were found through a regular beach survey carried out at the central-north coast of Rio de Janeiro State (22°55′S 42°30′W – 21°25′S 41°00′W) and at the north region of the coast of Rio Grande do Sul State (29°19′S 49°43′W – 31°26′S 51°07′W), Brazil. During beach surveys some cetaceans were found in advanced stage of decomposition not allowing precise taxonomic identification. On some occasions, only unidentified soft tissues were found (Figure 1). A small sample of about 10 g of the unidentified carcasses were collected and stored in alcohol 70% for further laboratorial analyses.

MATERIALS AND METHODS

Genomic DNA was extracted from four samples (GEMM 075, GEMM 088, GEMM 135 and GEMARS 1302) of unidentified baleen whale carcasses following a modified standard phenol–chloroform protocol described by Sholl *et al.* (2008).

Under continuous agitation, 30 mg of tissue were digested in 500 µl of lysis buffer (100 mM NaCl; 10 mM TRIS pH7,5), 25 µl of SDS 20%, 3 µl of Rnase (20 mg/ml^{-1}) and 3 to 5 µl of Proteinase XIV (20 mg/ml^{-1}) for 2 hours, subsequently disrupted inside a 1.5 ml microcentrifuge tube with a plastic rod, and incubated at 37°C for at least 12 hours. Following incubation, DNA extraction was carried out by the standard phenol–chloroform protocol (Sambrook *et al.*, 1989). DNA was diluted in 30 µl of sterile water.

Fig. 1. Figure showing images of two baleen whale carcasses stranded on the Brazilian coast and sampled for the present study: (GEMARS 1302) baleen whole carcass, lately identified as *Balaenoptera acutorostrata*, washed ashore on the coast of Tramandaí, Rio Grande do Sul State, southern Brazil, (GEMM 088) carcass later identified as *Balaenoptera edeni*, washed ashore on the beach from Restinga da Massambaba, Arraial do Cabo, Rio de Janeiro State, south-eastern Brazil.

The complete cytochrome *b* gene (1140 base pairs) was amplified using primers CBout1 (5′AATGAYATGAAAAR YCATCGTTG-3′) and CB-out2 (5′TCTTCCTTGAGTCTT AGGGAG-3′) (Cassens *et al.*, 2000). Amplifications were carried out in 50 ml reactions containing 250 ng to 1.0 mg of DNA, dNTPs (0.5 mM/ml), primers (0.3 pmol/ml), Taq DNA polymerase (Invitrogen, 0.04 U/ml) and amplification buffer under the following conditions: 94°C (1 minute), and 35 cycles at 94°C (1 minute), 55°C (1 minute) and 72°C (90 seconds). Four primers were used for sequencing: CB-out1 and CB-out2, which were used as external primers, and two internal primers: CB-in1 (5′-TTRTTRGATCCTGTTTC RTG-3′) and CB-in2 (5′-TGAGGACAAATATCATTYT GAG-3′) (Cassens *et al.*, 2000). The polymerase chain reaction (PCR) products were purified with 'GFXTM PCR DNA and Gel Band Purification' kit (Amersham Pharmacia), and both strands were sequenced in ABI Prism 3730 automatic sequencer. Sequences were edited with BioEdit Sequence Alignment Editor version 7.0 (Hall, 1999).

A blast search using the nucleotide–nucleotide basic local alignment (blastn) search tool was conducted using the sequences generated. A neighbour-joining tree was generated using the software Molecular Evolutionary Genetics Analysis (MEGA3) (Kumar *et al.*, 2004) adding eight sequences from GenBank from all species of baleen whales that occur on the coast of Brazil (Table 1) and two delphinids as outgroups (GenBank accession numbers EF488216 and X92526).

RESULTS

GEMM 075 and GEMARS 1302 showed 98% identity to one sequence of dwarf minke whale (*Balaenoptera acutorostrata*) (AP006468). Samples GEMM 088 and GEMM 135 showed 99% identity to sequences from Bryde's whale (*Balaenoptera edeni*) (X75583) and humpback whale (*Megaptera novaeangliae*) (AP006467), respectively. The magnitude of the genetic differences found (i.e. less than 2%) is consistent with expected intraspecific variation in cetaceans (e.g. Viricel & Rosel, 2012).

The neighbour-joining analyses corroborated the one found using blastn, showing that all carcasses analysed demonstrated a correspondence to species of *Balaenoptera* already recorded in Brazil (Figure 2). Our results are in agreement with the pattern of seasonal distribution of baleen

Table 1. GenBank seguences of mysticeti cetaceans used to compare with sequences obtained from carcasses.

Name and taxonomy	GenBank accession number
Blue whale (*Balaenoptera musculus*)	NC001601
Fin whale (*Balaenoptera physalus*)	NC001321
Sei whale (*Balaenoptera borealis*)	X75582
Bryde's whale (*Balaenoptera edeni*)	X75583
Antarctic minke whale (*Balaenoptera bonaerensis*)	X75581
Dwarf minke whale (*Balaenoptera acutorostrata*)	X7573
Humpback whale (*Megaptera novaeangliae*)	X75584
Southern right whale (*Eubalaena australis*)	DQ095153

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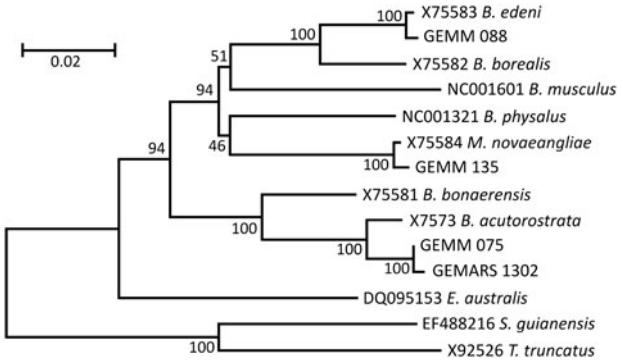

Fig. 2. Neighbour-joining tree between carcasses sequences and sequences from GenBank from all species of baleen whales that occur on the coast of Brazil and two delphinids as outgroup.

whales identified here, given that the three strandings occurred during winter and one during spring.

DISCUSSION

Dwarf minke whale is the most frequently stranded species of baleen whale on the Brazilian coast. The occurrence of *B. acutorostrata* in Brazil has been observed throughout the year, except during March, with peaks from June to September (Zerbini *et al.*, 1996, 1997).

In contrast to the highly migratory balaenopterid species, it is suggested that dwarf minke whales may spend the summer in mid-latitudes and feed in sub-tropical and warm temperate waters (Zerbini *et al.*, 1996). Hassel *et al.* (2003) sighted this species in January 2001 in eight different days and suggested that *B. acutorostrata* even take advantage of the prey species associated with the productive upwelling waters off Arraial do Cabo, Rio de Janeiro State.

The occurrence of humpback whales in Brazil is fairly well-understood. In the south-west Atlantic Ocean the wintering breeding grounds of *M. novaeangliae* are located on the north-eastern coast of Brazil, in the Abrolhos Bank (16°55′S 38°50′W) and its surroundings (Zerbini *et al.*, 2004). The breeding period of the whales in the Abrolhos Bank begins in June and ends in December of each year. Due to the coastal migratory movement on the south-eastern Brazil coast this species is frequently found stranded (Siciliano, 1997). Unlike the previous two species identified, *B. edeni* does not realize an extensive latitudinal migratory pattern. This species is frequently found in tropical and temperate waters, and is commonly sighted in coastal tropical waters of south-eastern Brazil, generally associated with other species of cetaceans, fishes and sea birds (Siciliano *et al.*, 2004; Moura & Siciliano, 2012). This species seems to take advantage of the trophic resources of this region associated with the coastal upwelling system present in this region, principally around Arraial do Cabo, Rio de Janeiro State (23°00′S 42°00′W).

The record of marine mammal strandings on coastlines helps to increase relevant information for conservation. Long term monitoring and detailed post-mortem analysis of strandings provide us with vital evidence of the causes of death and the ability to apply this knowledge for future conservation measures. This study reinforces the importance of using the DNA as a forensic tool to identify carcasses found on the beaches.

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REFERENCES

Carr S.M., Marshall H.D., Johnstone K.A., Pynn L.M. and Stenson G.B. (2002) How to tell a sea monster. molecular discrimination of large marine animals of the North Atlantic. *Biological Bulletin. Marine Biological Laboratory, Woods Hole* 202, 1–5.

Cassens I., Vicario S., Waddell V.G., Balchowsky H., Van Belle D., Ding W., Fan C., Lal Mohan R.S., Simões-Lopes P.C., Bastida R., Meyer A., Stanhope M.J. and Milinkovitch M.C. (2000) Independent adaptation to riverine habitats allowed survival of ancient cetacean lineages. *Proceedings of the National Academy of Sciences of the United States of America* 97, 11343–11347.

Geraci R. and Lounsbury V.J. (2005) *Marine mammals ashore: a field guide for strandings*. 2nd edition. Baltimore, MD: National Aquarium and Baltimore Press.

Hall A.T. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41, 95–98.

Hassel L.B., Venturotti A., Magalhães F.A., Cuenca S., Siciliano S. and Marques F.F. (2003) Summer sightings of dwarf minke whales (*Balaenoptera acutorostrata*) off eastern coast of Rio de Janeiro State, Brazil. *Latin American Journal of Aquatic Mammals* 2, 47–50.

Hermann B. and Hummel S. (1994) *Ancient DNA: recovery and analysis of genetic material from paleontological, archaeological, museum, medical and forensic specimens*. 2nd edition. New York: Springer-Verlag.

IUCN (2012) *IUCN Red list of Threatened Species*. version 2012.2. Available at: www.iucnredlist.org (accessed 22 January 2013).

Kocher T.D., Thomas W.K., Meyer A., Edwards S.V., Pääbo S., Villablanca F.X. and Wilson A.C. (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the United States of America* 86, 6196–6200.

Kumar S., Tamura K. and Nei M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* 5, 150–163.

https://doi.org/10.1017/S1755267212001157

Moura J.F. and Siciliano S. (2012) Stranding pattern of Bryde's whales along the south-eastern coast of Brazil. *Marine Biodiversity Records* 5, e73. doi: 10.1017/S1755267212000528.

Reynolds J. and Odell D. (1991) *Marine mammal strandings in the United States. Proceedings of the Second Marine Mammal Stranding Workshop*. NOAA Technical Reports, NMFS No. 98, 166 pp.

Sambrook J., Fritsch E.F. and Maniatis T. (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sholl T.G.C., Nascimento F.F., Leoncini O., Bonvicino C.R. and Siciliano S. (2008) Taxonomic identification of dolphin love charms commercialized in the Amazonian region through the analysis of cytochrome *b* DNA. *Journal of the Marine Biological Association of the United Kingdom* 88, 1207–1210.

Siciliano S. (1997) *Características da população de baleias jubarte (*Megaptera novaeangliae*) da costa brasileira, com especial referência aos Bancos de Abrolhos*. Master's thesis. Universidade Federal Rural do Rio de Janeiro, Rio de Janeiro, Brazil.

Siciliano S., Santos M.C.O., Vicente A.F.C., Alvarenga F.S., Zampirolli E., Lailson-Brito J., Azevedo A.F. and Pizzorno L.A. (2004) Strandings and feeding records of Bryde's whales (*Balaenoptera edeni*) in south-eastern Brazil. *Journal of the Marine Biological Association of the United Kingdom* 84, 857–859.

Viricel A. and Rosel P.E. (2012) Evaluating the utility of cox1 for cetacean species identification. *Marine Mammal Science* 28, 37–62.

Walsh M.Y., Odell D.K., Young G., Asper E.D. and Bossart G. (1990) Mass strandings of cetaceans. In Dierauf L.A. (ed.) *CRC handbook of marine mammal medicine: health, disease and rehabilitation*. Boca Raton, FL: CRC Press, pp. 673–683.

Weisburd S. (1984) Whales and dolphins use magnetic 'roads'. *Science News* 126, 389.

Zerbini A.N., Secchi E.R., Siciliano S. and Simões-Lopes P.C. (1996) The dwarf form of the minke whale, *Balaenoptera acutorostrata* Lacépède, 1804, in Brazil. *Reports of the International Whaling Commission* 46, 333–340.

Zerbini A.N., Secchi E.R., Siciliano S. and Simões-Lopes P.C. (1997) A review of the occurrence and distribution of whales of the genus *Balaenoptera* along the Brazilian coast. *Reports of the International Whaling Commission* 47, 407–417.

and

Zerbini A.N., Secchi E.R., Bassoi M., Rosa L.D., Higa A., Sousa L., Moreno I.B., Möller L.M. and Caon G. (2004) *Distribuição e abundância relativa de cetáceos na Zona Econômica Exclusiva na Região Sudeste-Sul do Brasil*. Série Documentos REVIZEE–Score Sul–IO-USP, 40 pp.

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