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SHORT NOTE

Mitogenomic insights into a recently described and rarely observed killer whale morphotype

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Abstract Identifying evolutionary divergent taxonomic units, e.g. species and subspecies, is important for conservation and evolutionary biology. The 'type D' killer whale, *Orcinus orca*, is a rarely observed morphotype with a pelagic, circumpolar subantarctic distribution, making dedicated research and therefore taxonomic study extremely difficult to date. In this study, we used DNA target enrichment hybridisation capture coupled to high throughput sequencing, to obtain the first DNA sequence from the only known museum specimen of this recently described morphotype. The high coverage, complete mitogenome sequence was compared to a previously published global dataset of 139 individuals, indicating that this type is highly divergent to all previously genetically sequenced killer whale forms. The estimated divergence time (390,000 years ago) from its most recent common ancestor with other extant killer whale lineages was the second oldest split within the killer whale phylogeny. This study provides the first genetic support of type D potentially being a distinct subspecies or species of killer whale, although further samples are needed to identify whether there is monophyly of mitogenome sequences and whether nuclear DNA also indicates reproductive isolation. These findings also highlight the value of natural history museum collections and new technologies to investigate the taxonomy of rare, cryptic or difficult to access species.

Keywords Mitogenome · Target enrichment capture · Ancient DNA · Killer whale

Introduction

The killer whale is one of the most widely distributed (and perhaps most universally recognisable) vertebrates in the ocean and one of the most intensively studied species of dolphin. Yet, remarkably, in the last decade, several new and often sympatric, morphological forms have been described (Pitman and Ensor 2003; Pitman et al. 2011), indicating that there is still much to learn about killer whale phylogenetics and ecology. Recently, a 'type D' or 'subantarctic' killer whale (Fig. 1) was described based on a single mass stranding 50 years ago and six recent at-sea sightings (Pitman et al. 2011); currently, it is considered the most morphologically distinct, but least well known of all the purported killer whale types.

On 13 May 1955, 17 killer whales stranded on Paraparaumu Beach, New Zealand (Baker 1983; Visser and Mäkeläinen 2000; Pitman et al. 2011). Photographs of the animals showed some unusual morphological characteristics, including an extremely small post-ocular eye patch

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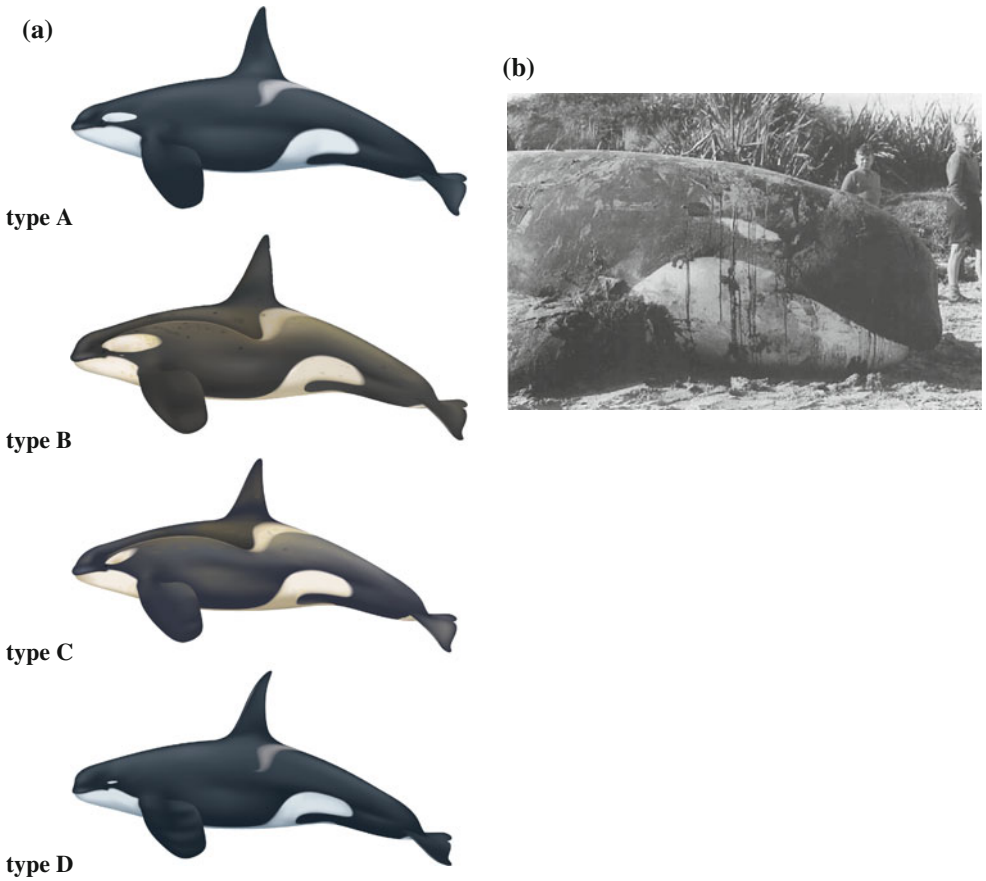

Fig. 1 Morphology of the subantarctic *type D* killer whale compared to adult males of Antarctic killer whale morphotypes, from Pitman et al. 2011; (not to scale). (**a**) *Type A* has the typical killer whale pigmentation patterning found globally, including in subantarctic and Antarctic waters; *type D* has the smallest post-ocular eye patch of any of these morphotypes, a more bulbous head, a narrower dorsal fin, and it lacks a distinct dorsal cape. *Yellow tinge* on *types B* and *C* results from diatoms. Illustrations by Uko Gorter (www.ukogorter.com). (**b**) Photograph of a type D killer whale from a stranding of 17 whales on 13 May 1955 at Paraparaumu Beach, New Zealand (photo courtesy Museum of New Zealand) from which our tissue samples were collected. (Color figure online)

and bulbous head (Visser and Mäkeläinen 2000; Pitman et al. 2011). It was over half a century later when killer whales with these particular features were reported again, when six separate at-sea sightings were photo-documented between 40°S and 60°S, suggesting a circumpolar range in subantarctic waters (Pitman et al. 2011). Type D killer whale is an offshore inhabitant of some of the most turbulent oceans in the world, which explains the lack of sighting and stranding records to date, and also makes dedicated research extremely difficult. Consequently, no tissue samples have been collected from free-ranging individuals to allow molecular analysis that would help resolve its phylogenetic relationship to the other killer whale lineages.

Here, we have used ancient DNA methods to sequence the complete mitochondrial genome of a specimen from the 1955 New Zealand stranding. DNA was independently extracted, amplified and sequenced from two different tissue types, which provided identical sequences. We then used Bayesian phylogenetic inference to compare this sequence to an extensive global dataset of killer whale mitogenomes (Morin et al. 2010) and reconstruct an evolutionary history of this distinctive morphotype.

Materials and methods

We extracted DNA from a piece of dried soft tissue on the skull and from approximately 0.5 g of powdered tooth from one of the Paraparamu stranded specimens (#1077, Museum of New Zealand Te Papa Tongarewa, Wellington, NZ). DNA was extracted and purified from powdered tooth as per reference (Foote et al. 2009) and from the dried tissue using the Qiagen DNeasy kit following the manufacturer's guidelines. A blank extraction was included with each protocol to monitor for contamination. To generate the complete mitogenome sequence, we used DNA target enrichment hybridisation capture coupled to high

throughput sequencing, using the protocol presented by Maricic et al. (2010). Illumina sequencing libraries were built on the DNA extracts using NEBNext (Ipswich, MA, USA) DNA Sample Prep Master Mix Set 1 following Meyer and Kircher (2010). Libraries were subsequently index amplified for 15–20 cycles using Phusion High-Fidelity Master Mix (Finnzymes) in 50 µL reactions following the manufacturers guidelines. The libraries were then purified using MinElute PCR purification kit (Qiagen, Hilden, Germany) and pooled equimolarly into a total of 2 µg, at which point they were ready for subsequent target enrichment. To generate the bait for target enrichment, high-quality killer whale DNA extract, from the blood of a captive Norwegian killer whale, for which we had genomic DNA of high purity, molecular weight and concentration, was amplified for four overlapping long-range PCR products using primers LR2.1, LR2.2, LR3 and LR4 encompassing the whole mitogenome, following Morin et al. (2010). Subsequently, these amplicons were converted into bait following Maricic et al. (2010), after which target enrichment proceeded on the pooled libraries following Maricic et al. (2010). The DNA concentration of the library eluted post-capture was measured using a 2100 Bioanalyser (Agilent Technologies) and then sequenced in subpartitions of single channels on an Illumina HiSeq 2000 platform using SR 100 bp chemistry.

Illumina HiSeq 2000 reads were filtered with AdapterRemoval (Lindgreen 2012), to remove adapter dimers as well as low quality stretches at the 3′ ends. Filtered reads were then mapped to a reference killer whale mitogenome (GU187176.1) using BWA version 0.5.9 (Li and Durbin 2009), requiring a mapping quality of >25. Clonal reads were collapsed using the rmdup program of the Samtools (version 0.1.18) suite (Li et al. 2009). Ambiguously mapped reads were also filtered out using Samtools and controlling for XT, XA and X1 tags. Consensus mitogenome sequences were then reconstructed using bam files, which were aligned in GENEIOUS (Biomatters Ltd.).

We generated a time-calibrated Bayesian inference phylogeny to reconstruct the relationship of our type D killer whale samples to a global dataset of killer whales (see Online Resource 1). The program BEAST v1.6.2 (Drummond and Rambaut 2007) was used to co-estimate mitogenome divergence times and a tree topology under a Bayesian framework. We analysed the entire mitogenome sequence as it has been shown to provide highest posterior probabilities for topology and date estimates for killer whales (Duchene et al. 2011), and used the optimal BIC-selected mutation model using the program J-ModelTest v. 2.1 (Darriba et al. 2012). We used the Relaxed log-normal clock, a Yule speciation process and a single TMRCA prior for all killer whales based on previous analyses (Morin et al. 2010; Duchene et al. 2011). We performed 10^6 MCMC chains checked for performance analysis in the program TRACER v. 1.5.0 (Drummond and Rambaut 2007).

Results and discussion

Sequence reads were trimmed, filtered and mapped to a reference mitochondrial genome, resulting in >5× read depth across all 16,386 positions in the sequence generated from the tooth sample and >5× read depth across 16,328 positions in the sequence generated from the soft tissue sample (GenBank Accession Number TBC). The two sequences matched 100 % across all sequenced positions indicating no nucleotide misincorporations due to DNA damage or sequencing errors. The Illumina sequencing also matched Sanger sequencing of the hyper variable region of the D-loop performed on the tooth sample. The agreement between independently generated sequences of the same specimen and the discordance with the mitogenome of the specimen used for bait in the capture indicates no bait fragments were sequenced.

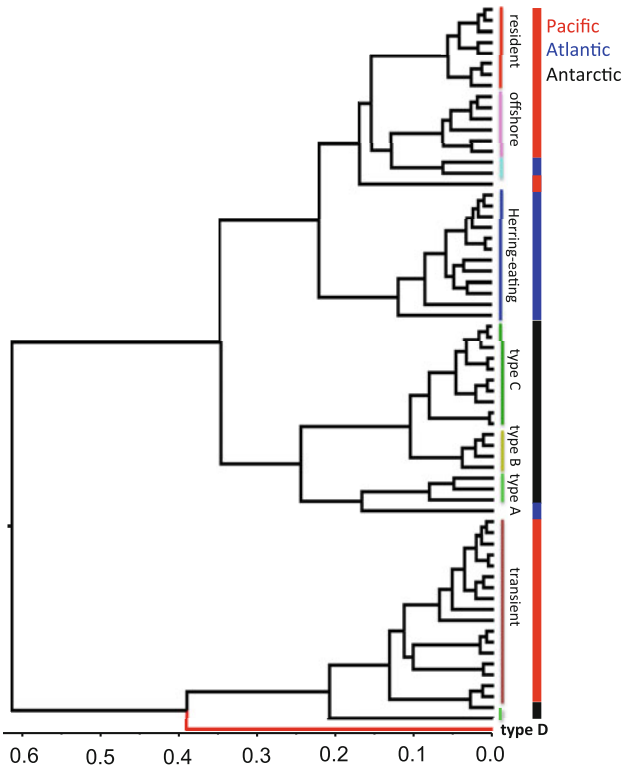

Fig. 2 Bayesian complete mitogenome phylogenetic tree of 67 unique killer whale haplotypes derived from 140 samples. The branch representing the *type D* killer whale sequence is *coloured red*. The *scale bar* indicates divergence times in million years BP. The *thin coloured vertical lines* identify the dominant killer whale ecotype or population within each clade, whilst the *thick vertical lines* indicate the ocean basin that each haplotype was detected. (Color figure online)

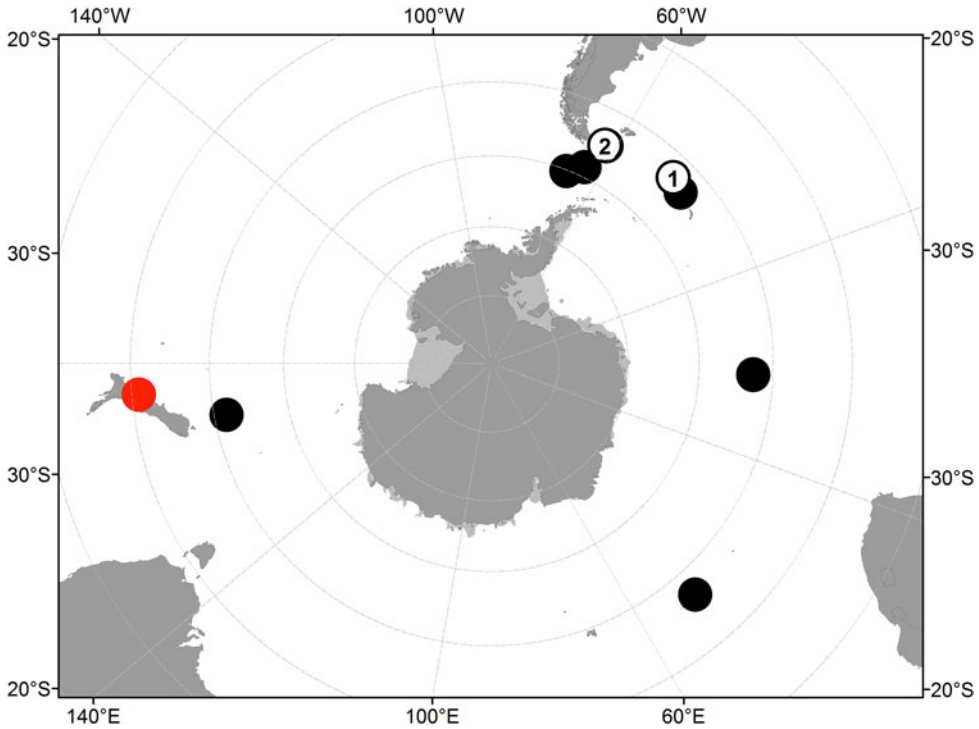

Fig. 3 Location of the 1955 stranding of 17 type D killer whales on Paraparaumu Beach, New Zealand (*red circle*) and nine photographically confirmed at-sea sightings of this morphotype around Antarctica. The six sightings reported in Pitman et al. (2011) are shown by *closed circles*; three new sightings since Pitman et al. (2011) are indicated with *open circles* and include: (1) 28 Nov 2011: 52°56′S, 45°30′W, 30 individuals, J-P. Sylvestre personal communication; (2) 2 Nov 2012: 54°49′S, 62°08′W, nine individuals, J. Watts personal communication; (3) 2 Nov 2012: 54°50′S, 62°23′W, three individuals, J. Watts personal communication. The last two encounters occurred only a few miles apart in Drake Passage and are indicated with a '2' in the *circle*. (Color figure online)

The type D lineage was highly divergent from any other previously sequenced killer whale, sharing its most recent common ancestor (99 % pairwise identity) with the clade containing the North Pacific marine mammal-eating 'transient' ecotype (Fig. 2). The estimated divergence date of approximately 390,000 years ago is the second oldest divergence within our killer whale phylogeny. The estimated timing of this split falls within the interglacial period, which occurred between 424,000 and 374,000 years ago (Marine Isotope Stage 11), the warmest interglacial period of the last 500,000 years (Howard 1997). The 95 % highest posterior density interval (HPDI) was 230,000–580,000 years ago, and so, the diversification may have occurred during the adjacent glacials. The dramatic changes in global sea level and ice sheet coverage during these glacial cycles within the HPDI (Huybrechts 2002) are thought to promote speciation through facilitating range expansion during inter-glacials followed by long periods of isolation among populations within different refugia during glacials (Rogers 2007).

Whilst mitogenomes from more samples and nuclear DNA analysis are required to confirm that type D is reproductively isolated from other killer whale types to qualify as a new species under the Biological Species Concept, in the absence of this data, the deep divergence within the killer whale phylogeny, divergent morphology, and, apparently, overlapping at-sea range with all of the other Southern Ocean types provide accumulating lines of evidence that suggest type D is potentially a new species or subspecies of killer whale (see De Queiroz 2007; Morin et al. 2010).

Three additional type D killer whale sightings collected during 2011–2013 (Fig. 3) are consistent with the previous suggestion of a circumpolar, subantarctic range, north of the Polar Frontal Zone (PFZ) (Pitman et al. 2011). The PFZ is an important oceanographic barrier driving speciation in many taxa (Rogers 2007) and may be an effective barrier to contact and gene flow between type D and the morphologically divergent types A, B and C killer whales, which are normally found in more southerly, Antarctic waters. The putatively adaptive amino acid changes in the *cytochrome b* gene found in the types B and C killer whales that inhabit the pack-ice around the Antarctic continent (Foote et al. 2011) were not present in our type D mitogenome sequence.

Photographs from the 1955 New Zealand mass stranding of this morphotype show that at least one individual had a cookie cutter shark (*Isistius* sp.) bite mark on its flank (Dwyer and Visser 2011). Cookie cutter shark bites were not observed in any of the photographed encounters in

subantarctic waters of type D killer whales that we or Dwyer and Visser (2011) examined. These observations suggest that the museum specimen genetically analysed here, which originated from the 1955 mass stranding in New Zealand, where cookie cutter shark bites on cetaceans are common (Dwyer and Visser 2011), may have been outside the normal northerly limits of the range of this morphotype. However, further work including satellite tagging is required to better understand the geographic range of this morphotype.

Obtaining tissue samples and other biological data from rare or geographically remote populations for use in population genetic or phylogenetic studies can be logistically challenging and can leave gaps in our understanding of species diversity and evolutionary history. Several recent studies have highlighted how ancient DNA methods can provide new insights into the evolutionary history or taxonomy of a species using natural history museum specimens and archaeological finds (e.g. Briggs et al. 2009; Kirchman et al. 2010). Similarly, another recent study highlighted the importance of DNA identification of beach-cast animals as part of a well-co-ordinated stranding network (Thompson et al. 2012). This case study highlights how, in some cases, ancient DNA analysis of specimens already within natural history museum collections can provide a means to resolve some of these phylogenetic uncertainties. These museums, if properly maintained, will continue to remain valuable sources of biological discovery as new and emerging molecular approaches become available.

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