Ecotype Origin of an Entangled Killer Whale (*Orcinus* orca) Identified with Remnant mtDNA

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On June 26, 2022, a dead killer whale (*Orcinus orca*) was found 48 km off the coast of Newport, Oregon entangled in presumed recreational Dungeness crab (*Metacarcinus magister*) fishery gear, a crab pot and line. The line was wound around the peduncle, proximal to the fluke (Figure 1). A recreational angler photographed the whale and submitted a set of images of the animal's ventral side to an online forum (http://www.ifish.net). Identifying the individual was not possible from these images, as the visible features did not include those commonly used in killer whale photo identification (Bigg et al., 1990; Young et al., 2011). The Oregon Marine Mammal Stranding Network (OMMSN) was informed and promptly notified the U.S. National Oceanic and Atmospheric Administration (NOAA) Fisheries Service, leading to aerial and seaborne responses by the U.S. Coast Guard.

The carcass was not found off Newport but instead re-sighted on July 7, 2022, off the coast of Bandon, Oregon—over 160 km south—by another recreational angler. By this point, the carcass had undergone substantial taphonomic change. The primary posterior elements were degraded down to the skeleton; the crab pot and line were still attached to the whale (Figure 1b, 1c). The second reporting party cut the line and trap free from the carcass and turned the gear in to the Port of Bandon (https://www.portofbandon.com/). OMMSN recovered the gear and transported it to Oregon State University's (OSU) Hatfield Marine Science Center (HMSC) in Newport. The crab pot measured 89.5 cm in diameter, 25.0 cm high, and had a mesh size of 6.0 cm. There were no identifiable serial markers on the trap or buoys due to exposure and fouling (Figure 1).

[Figure 1 here]

Figure 1a. A map of the Oregon Coast with red points denoting the sighting locations and dates of the dead entangled killer whale. **1b.** The carcass was first sighted offshore of Newport, Oregon on June 26, 2022. Photo taken by Don Grim. **1c.** The killer whale carcass was last observed on July 7, 2022 offshore Bandon, Oregon, where the debris was removed by the reporting party. Photo by Mark Eason. **1d.** A speculative life illustration of the killer whale with the site of entanglement circled in red. Illustration by Charles Nye.

The public and the NOAA regional office expressed an interest in identifying the ecotype (a behaviorally and morphologically distinct sympatric group within a species) of the carcass (Bigg et al., 1990; Bruyn et al., 2013; Ford et al., 1998). Killer whales that inhabit the coastal waters of the Northeast Pacific are relatively well-documented from both traditional identification methods (i.e., distinguishing physical attributes, acoustics, and morphology) and genetic markers (Baker et al., 2018; Hoelzel et al., 1991; Zerbini et al., 2007; Young et al., 2011; Parsons et al., 2013). Several ecotypes and populations occupy this region of the ocean, including Northern Resident killer whales (NRKWs), Southern Resident killer whales (SKRWs), Transient (or Bigg's) killer whales (TBKWs), and Offshore killer whales (OSKWs) (Bigg et al., 1990; Hoelzel & Dover, 1990; Ford et al., 1998; Dalheim et al., 2008). In the U.S., two Pacific killer whale groups are recognized as separate management units: the Alaskan TBKW AT1 population, which is considered Depleted following the *Exxon Valdez* oil spill of 1989, and the SRKWs, which are Endangered under the U.S. Endangered Species Act (ESA) (Carretta et al., 2019; Muto et al., 2019). In Canada, most killer whale populations are defined as Threatened under Schedule 1 of the Species at Risk Act, with SRKWs considered Endangered (Fisheries and Oceans Canada, 2017).

Killer whale ecotypes are distinguishable using a fragment of the mitochondrial genome known as the control region, or "d-loop" (Zerbini et al., 2007; Parsons et al., 2013; Baker et al.,

2018). Although no tissue samples had been collected from the dead whale, we considered it likely that prolonged contact with the crabbing line would have inundated sections of the gear with recoverable DNA. Given the prolonged environmental exposure and decay of the body, we hypothesized that any usable genetic material would likely originate from the mitogenome, as is common in these environments (Bylemans et al., 2018).

Here, we present evidence for the ecotype origin of the entangled killer whale using investigative molecular methods. The crab pot and line were measured and photographed at the OMMSN necropsy lab at HMSC. Photos of the entangled carcass in situ were cross-referenced to locate sections of the line that were near or in direct contact with the deceased killer whale (Figure 1c, 1d). Using further visual and olfactory assessments, a ~5 cm portion of suspected organic material, along with a small portion of the line, was peeled off the gear with sterilized forceps and stored in a 10 mL glass scintillation vial.

We initially employed a metabarcoding approach to discern if any mtDNA was recoverable from the line sample, regardless of the species of origin. Genomic DNA was extracted from two >0.01 g subsamples using a QIAGEN DNeasy Blood and Tissue kit to the manufacturer's specifications. Sequencing was conducted on an Illumina MiSeq platform (SCR_016379) at OSU's Center for Qualitative Life Studies (CQLS). Following laboratory protocols detailed by Closek et al. (2018), we confirmed the presence of killer whale mitochondrial DNA by first PCR amplifying a 313-bp fragment of the common metabarcoding locus, cytochrome C oxidase subunit I (COI) (Leray et al., 2013). Amplicon sequence variants (ASVs) were quality-controlled and aligned using the program DADA2 in the CALeDNA Anacapa Toolkit (Callahan et al., 2016; Curd et al., 2019).

Taxonomic information was assigned to each ASV from a BLAST query of the full NCBI GenBank database, with any ASV below 5% of the average read count across the entire dataset being removed. Identifiable ASVs were secondarily validated using the Barcode of Life Data System (BOLD) database (Ratashingham & Herbert, 2007). From this exploratory step, we were able to confirm the presence of killer whale mtDNA; the associated ASV was a 100% match to the mtCOI sequence of killer whale ecotypes associated with the greater Northeast Pacific region (Filavota et al., 2018). Two additional taxa, gooseneck barnacle (*Lepas pectinata*) and a genus of rotifer (*Synchaeta spp*.), were also identified from these samples (Figure 2). We attribute the sequence abundance of these additional taxa, particularly of *L. pectinata*, to fouling on the crab pot and line that occurred during the gear's prolonged residence at sea.

[Figure 2 here]

Figure 2. Read abundances belonging to amplicon sequence variants (ASVs) identified to the species level in two suspected samples of killer whales taken from the entanglement line. The target 313-base-pair fragment of cytochrome C oxidase subunit I (COI) was amplified using degenerate metabarcoding primers designed by Leray et al. (2013).

Additional PCR assays were then conducted, targeting a ~690 base-pair fragment of the cetacean mtDNA control region (d-loop 1.5-8) which encompasses key informative loci for discriminating killer whale ecotypes and populations (Morin et al., 2010). The PCR products were purified and Sanger sequenced in the forward and reverse directions on an ABI3730xl platform. Only the second subsample was successfully amplified and sequenced for this locus, which is referred to hereon with its NCBI accession as OR661229 HMSC. Alignment and quantitative treatments for the resultant data were performed using the software package Geneious Prime (Version 2021.1.1) and a comprehensive dataset of unique killer whale mtDNA sequences (haplotypes) published by Zerbini et al. (2007) and Morin et al. (2010). We used a

Tamura-Nei distance for the mtDNA sequences, with a neighbor-joining tree (bootstrap resampling, n = 9,999) to visualize genetic distances (Figure 3).

[Figure 3 here]

Figure 3. A bootstrapped neighbor-joining tree (mid-point rooted) of killer whale mtDNA control region sequences generated using Tamura-Nei distance (resampled n = 9,999). The values shown are bootstrap values (% replicates that resolve to the depicted identity). Haplotypes in boxed in gray are Resident and Offshore killer whales; haplotypes boxed in blue are Transient/Bigg's killer whales. The sequence from the entangled killer whale described in this paper is positioned at the bold blue text as "OR661229 HMSC." Additional mtDNA sequences used were sourced from Morin et al. (2010) and Zerbini et al. (2007).

The d-loop 1.5-8 control region sequence amplified from the entangled killer whale was a 100% match to the published mitogenome of the TBKW haplotype ENPTSEA2 from the Northeast Pacific (Morin et al., 2010). All TBKW haplotypes cluster closely at nearly 98% bootstrapped confidence; haplotypes belonging to the other primary ecotypes are represented in a separate clade from the TBKWs (Figure 3). Comparing OR661229 HMSC to the SRKW haplotype SR, there are 7 variable nucleotide site differences in the alignment in addition to the apparent phylogenetic distance (Supplementary Table 1; Figure 3). We consider the results of the phylogenetic reconstruction sufficient to conclude that the entangled individual was a TBKW and not a SRKW, with high confidence it was of the ENPTSEA2 haplotype. Visible ventral markings from the entangled TBKW suggest it was a young male (Figure 1b) but we have been unable to confirm this using standard molecular markers for sex identification, presumably due to the degradation of the nuclear DNA (Bylemans et al., 2018).

Our findings demonstrate both the diagnostic capabilities of genetic sampling and the surprising residency of recoverable mtDNA from anthropogenic debris. mtDNA barcoding has been used in other wildlife forensics applications, from identifying endangered taxa traded in

markets to shark species from bite wounds (Baker 2008; Kraft et al., 2021; Lee et al., 2021). Genetic identification of marine mammal carcasses is standard for U.S.-based stranding networks, but we stress there may be added value from genetic analysis of marine debris associated with marine mammal entanglements, particularly in helping to assign an anthropogenic mortality event to ecotypes or Distinct Population Segments (Baulch and Perry, 2014; Carretta et al., 2021).

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Supplementary Table 1. A variable sites table of the killer whale control region haplotype alignments, with the entangled killer whale's sequence highlighted in yellow as the basis for comparison. Variable nucleotides are highlighted in blue.

Genbank Code / Haplotype	Corresponding KW Ecotype	6	133	147	276	284	304	392	409	458	503	505	542	626
OR661229 HMSC	Transient/Bigg's	:	T	A	G	T	A	C	G	T	T	C	C	T
GU187162.1 ENPTSEA2	Transient/Bigg's	:	.	.	.	.	.	.	.	.	.	.	.	.
DQ399075.1 NT1	Transient/Bigg's	:	.	G	A	.	.	.	.	.	.	.	.	.
DQ399076.1 NT2	Transient/Bigg's	:	C	G	.	.	.	.	.	.	.	.	.	.
DQ399080.1 GAT 2	Transient/Bigg's	:	.	G	.	.	.	T	.	.	.	.	.	.
DQ399081.1 GAT	Transient/Bigg's	:	.	G	.	.	.	.	.	.	.	.	.	.
DQ399082.1 AT1	Transient/Bigg's	:	.	G	.	.	G	.	A	.	.	.	.	.
DQ399077.1 SR	Resident (Southern)	A	.	G	.	.	.	T	A	C	C	.	T	.
DQ399074.1 NEWR	Resident (Other)	:	.	G	.	C	.	T	A	C	C	.	T	.
DQ399078.1 NR	Resident (Northern)	A	.	G	.	.	.	T	A	C	C	T	T	.
DQ399079.1 OFF	Offshore	:	.	G	.	.	.	T	A	C	C	.	T	C