Identification by Histological and Microsatellite Analyses of a Stranded Beaked Whale as that Struck Previously by a Jetfoil Operating in the Sea of Japan

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Histological and genetical studies were made to determine the identities of a large marine mammal struck by a jetfoil operating for the Sado Line (Niigata-Ryotsu route) in the Sea of Japan and a female Stejneger's beaked whale stranded on Kasashima beach, ca. 100 km from the collision point, one day after the collision. Tissue remains (comprising skin, muscle and bone) taken from the intake pipe of the jetfoil were histologically identical to similar samples from the stranded specimen. rDNA typing for species identification showed an identical sequence of 107 bp, and microsatellite analysis demonstrated that the genotypes of both animals were identical at three loci. Accordingly, the animal struck by the jetfoil and that stranded on the beach are considered to be one and the same individual.

Key words: cetacean collision, cetacean skin, rDNA, microsatellite analysis, Mesoplodon stejnegeri

The identity of an unknown object struck by a jetfoil operating for the Sado Line in the Sea of Japan, has previously determined by only a single histological study, using a portion of muscle lodged in the intake (suction) pipe of the boat. That examination revealed that the animal struck was most likely a species of whale.

Jetfoils commenced operations in Japanese waters for the Sado Line on 1 May, 1977. Since then, nine collisions have been reported by the Sado Line Company to the present time.

On 2 March, 1997, the jetfoil “Tsubasa” (meaning wing), travelling on the Niigata-Ryotsu route collided with an unknown object, damage to the boat being minimal. However, careful examination of the hull after docking the next day, disclosed a small mass of muscle with skin attached, apparently from the struck animal, lodged in the intake pipe near the stern. This material was preserved for histological examination.

The day after the above collision, a female beaked whale Mesoplodon stejnegeri ca. 5 m in total length and 1.5t in weight, was stranded on Kasashima beach, Kashiwazaki City, Niigata Prefecture (Fig. 1). This specimen was subjected to macroscopical examination by several specialists at the spot. During this investigation, complex major fractures of the IVth and Vth thoracic vertebrae and associated ribs were encountered. In addition, despite no obvious major wounds being apparent, severe internal hemorrhaging was found in the abdominal cavity. This was the first instance of this kind of serious damage amongst the many stranded cetaceans examined from the Niigata coast.2-7 Accordingly, the question arose as to whether or not the stranded beaked whale might have been the object struck by the jetfoil the day before, although the distance from the collision point on the Niigata-Ryotsu route to Kasashima beach is ca. 100 km.

Therefore, in addition to histological examination of muscle samples, including skin and bone fragments, from both specimens, genetic identification was also carried out, the validity of such being suggested and supported by following various reports, such as Pastene et al., Goto and Pastene, Abe et al., Valsecchi and Amos, Richard et al., Buchanan et al., Shinohara et al., Palsboll et al. Accordingly, three microsatellite loci were analyzed and a comparison of ribosomal ribonucleic acid gene (rDNA) typing was made.

Materials and Methods

Histology

Ten percent formalin preserved blocks of both struck (X) and stranded (W) specimens were refluxed in Bouin’s solution, and subsequently dehydrated through a graded alcohol series, embedded in paraffin and cut at 8 μm thickness. The sections were stained chiefly with Masson-Goldner trichrome (MG), and Mayer’s hematoxylin-eosin double stain (HE) for examination under a light microscope.

Genetic Identification

Genomic DNA was isolated from the two samples, X (see above) and W (skin only), by proteinase K digestion and phenol/chloroform extraction.

For microsatellite marker typing, three sets of primers were used, following Palsboll et al., that detected the
Fig. 1. A female Stejneger's beaked whale *Mesoplodon stejnegeri* (ca. 5 m in total length) stranded on Kasashima beach, Kashiwazaki City, on 3 March, 1997.

The primer sequences used in this study were as follows: GATA417 forward: 5'-CTGAGATAGCTTACATGGG-3', reverse: 5'-TCTGCTCAGGAAAATTTTCAAG-3'; GATA028 forward: 5'-CGCTGATAAGTGCTCTAGG-3', reverse: 5'-AAAGACTGAGATCTATAGTTA-3'; GATA098 forward: 5'-TGTACCCTGATGGATAGATT-3', reverse: 5'-ATGTCTCTCTCACAACACC-3'.

The polymerase chain reaction (PCR) was carried out in a 15 μl reaction mixture containing 50-80 ng template DNA and 0.75 U of AmpliTaq Gold™ DNA polymerase (Perkin-Elmer). Amplification was performed in a Perkin Elmer Model 9700 thermocycler using the following conditions: one cycle for 9 min at 95°C, 40 cycles of denaturation of 1 min at 94°C, annealing for 30 s at 47°C for GATA028, 50°C for GATA417 and GATA098, extension for 30 s at 72°C, and one cycle for 10 min. at 72°C. After amplification, PCR products were mixed with an equal volume of formamide loading buffer (formamide 95%, EDTA-3Na 20 mm, BPB 0.005%, xylene cyanol 0.005%), heat denatured and electrophoresed on 8% (GATA417 and GATA028) or 12% (GATA098) acrylamide-bisacrylamide (29:1) denaturing sequencing gel (8 M urea, 1 mm thick). DNA bands were visualized by silver staining.

Table 1. DNA samples from other 6 individuals of the Stejneger's beaked whales *Mesoplodon stejnegeri* used in this study

<table>
<thead>
<tr>
<th>Date</th>
<th>Location</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 Apr., 1996</td>
<td>Kakizaki Town, Niigata Pref.</td>
<td>4.66 m in length</td>
</tr>
<tr>
<td>25 Apr., 1996</td>
<td>Oogata Town, Niigata Pref.</td>
<td>5.30 m</td>
</tr>
<tr>
<td>27 Feb., 1995</td>
<td>Iwami Town, Tottori Pref.</td>
<td>4.99 m</td>
</tr>
<tr>
<td>1 Feb., 1996</td>
<td>Tsuruoka City, Yamagata Pref.</td>
<td>5.10 m</td>
</tr>
<tr>
<td>5 Jan., 1993</td>
<td>Rishiri Town, Hokkaido</td>
<td>4.30 m</td>
</tr>
<tr>
<td>3 Mar., 1993</td>
<td>Noh Town, Niigata Pref.</td>
<td>4.80 m</td>
</tr>
</tbody>
</table>

These loci have been revealed as being highly polymorphic in minke whales from the western North Pacific. There is, however, little microsatellite data for Stejneger's beaked whales. Therefore, DNA samples from 6 other individuals of this species (Table 1) stranded on the coast of Sea of Japan (made possible through the courtesy of Dr. M. Goto, Institute of Cetacean Research, Tokyo) were here genotyped for the three loci.

rDNA typing for species identification was performed with direct sequencing. Extracted DNA was subjected to a hot started PCR assay with AmpliTaq Gold™ as described above for microsatellite typing using a pair of primers, except annealing took place at 60°C. The primers, A: AUCUAGGUAGGUUCCUC; B: GUAAGCGAAUGAUUGAGG, generated a part of the 28S rRNA gene, including the variable loop region.
flanked by conservative sequences. The PCR products were purified to remove dNTPs and primers using NANOSEP™ microconcentrators (Pall Filtron) and directly sequenced. Sequencing was accomplished using the taq-base cycle sequencing system incorporating fluorescent dideoxy terminators (ABI PRISM3 10 Genetic Analysis System).

**Results**

**Light Microscopy**

No clearcut differences were encountered between the jetfoil-struck (X) and stranded (W) specimens, except for a weaker affinity for dyes and heavier fractures in the latter. Accordingly, further histological description and attached figures were based exclusively on sample X. The skin, comprising epidermis and dermis, consisted of (1) stratum corneum (stratified epithelium), (2) one to two layers of s. granulosum, (3) a thick layer of s. spinosum and (4) s. basale, which bordered (5) the dermis. The dermis was composed of adipose tissue, a large amount of collagenous fibers and a few reticular fibers (Fig. 2).

Several dermal papillae were projected toward the epithelium. S. corneum showed no true keratinization and exfoliation, the surface appearing to be rather smooth. The s. granulosum cells, spindle or long ovoid in shape, contained an ovoid or long ovoid nucleus (red tinted) and melanin granules, which seemed to have been transported from the melanocytes occurring in the s. basale. The cytoplasm was deeply stained with orange G.

S. spinosum cells, ovoid in shape, were positioned vertically against the surface (s. corneum), and also contained melanin granules located near the apical portion of the nucleus (stained pale with dyes). Development of intercellular bridges (prickles) were clearly apparent. The cytoplasm was stained red, owing to the presence of rich filamentous components, probably tonofilaments. The prickles projecting from the cells comprised presumed desmosomes (Fig. 3).

On the other hand, the columnar s. basale cells were disposed vertically to the basement membrane at the dermo-epidermal junction. The cell outline (border) was indistinct, although containing a large amount of closely-concentrated melanin granules. (A small portion of the latter have been expelled from the cell.) Nuclear chromatin showed a strong affinity for dyes, such as hematoxylin and fuchsin (Fig. 4).

In the dermis, consisting mainly of collagenous stroma, numerous capillaries occurred near the dermo-epidermal junction. Just beneath this region, well developed adipose tissue was apparent in association with MG-stained collagenous fiber strands (deeply red) and reticular fibers (purple) (Fig. 5).

Muscle fiber strands were fragmented (by a supposedly strong impact), no muscle spindles with large adipose droplets being found in either sample mass (Figs. 6a, b).

So-called blubber, consisting entirely of hypodermis, was constructed from connective tissue fibers without muscle.
Bone fragment sections revealed both spongy and trabecular bones. Therefore, a lamellar structure and lacunae containing osteocytes were observed as expected (Fig. 7). Weak calcified portions, including fibrous connective tissue and a considerable amount of adipose tissue, occurred randomly. Fibrous strands, showing a strong affinity for red dyes (such as ponceau de xylidine and eosin), were also apparent, along with an osseous organic ground substance (=bone matrix) stained light green. The margin of the Haversian-like cavity, (i.e., the margin of the central cavity), had a brush border-like structure (Fig. 8).

Genetic Identification

Four discrete alleles (A₁–A₄) were observed for GATA417, 2 (B₁–B₂) for GATA028 and 4 (C₁–C₄) for GATA098 in 7 Stejneger's beaked whales, including sample W (Figs. 9, 10). The genotypes of sample X were identical to those of W at three loci.

In addition, the two samples (X and W), showed identical sequences of 107 bp, indicating that the X was cetacean.

Discussion

A recent report following histological examination of a muscle mass, including bone fragments, from a large unknown mammal struck by a jetfoil on 31 October, 1994, revealed the animal in question to be a cetacean.¹ This report was the first attempt to determine the identity of...
Fig. 7. Section of spongy bone (X), showing lamellar structure and lacunae with osteocytes (▲). HE stain. × 400.

Fig. 8. Haversian-like cavities (X), showing a brush border-like margin (▲). MG stain. × 400.

Fig. 9. Microsatellite genotyping with GATA417, GATA028 and GATA098 probes.

PCR amplification and electrophoresis were performed as described in microsatellite marker typing. Allele designation at each locus made temporarily with alphabet-No., indicated to left of each panel. Genotypes of X and W shown at bottom of each sample lane. Lane 1–6: Stejneger’s beaked whale; W: Stejneger’s beaked whale stranded on Kasashima Beach; X: remains of marine mammal struck by jetfoil.
animals struck on five different occasions by the jetfoils scheduled on the Sado Line between Niigata and Ryotsu (Sado Island) in the Sea of Japan.

To the present time, nine separable incidences of jetfoil collisions with undetermined floating objects have been recorded by this Line. In addition to a previous report,1) which described light and electron microscopy of muscle and bone, the present study adds a light microscopic examination of skin as was described by Simpson and Gardner,17) although very rare existence of muscle spindles, described by Honma et al.,1) could not be detected here.

In the present study, the tissue design and architecture of the skin samples, in addition to the spongy bone structure, revealed similar structures, consistent with that of a Stejneger's beaked whale.

In addition, two aspects of genetic examination were carried out using tissue remains of both samples, which were suitable for DNA extraction because of comprising a large number of epidermal cell nuclei. Thus, by microsatellite analyses and rDNA typing, the two samples were determined as representing the same Stejneger's beaked whale.

References