Investigating the Usability of DNA from Sloughed Skin for Individual Identification in Humpback Whales *(Megaptera novaeangliae)*

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Abstract

Obtaining genetic samples from cetaceans can be logistically challenging. Various methods of DNA collection for cetaceans have been utilized in the past including biopsy darting (invasive, strict permit regulations), fecal sampling (difficult to find, reduced quality DNA) and sloughed skin collection. In this study, we demonstrate that sloughed skin collection provides a non-invasive, low-cost means of acquiring high quality DNA for individual identification of humpback whales (*Megaptera novaeangliae*). Using a small subset of a large (> 1,500 samples) long-term sloughed skin dataset, we compared genotypes from multiple samples of known individuals (confirmed from field observations and photo-ID) that had been seen over multiple field seasons. For all cases in which we obtained more than one skin sample from a whale identified in the field as the same individual, we confirmed that the genotypes were identical. We used a panel of 14 microsatellite markers and M13 universal primer labels to compare genotypes of samples collected from mothers and their calves and demonstrated that, in all confirmable cases, known mothers shared one-half of their genotype with their observed calves. M13 universal primer labels enabled us to determine genotypes at a fraction of the price required of fluorescent-labeled dye primers. Careful analysis of genotypic data from sloughed skin allows us to address questions about humpback whale social dynamics, reproductive success, ecology and life history. In the future, these techniques can be applied to the larger humpback whale dataset and to other cetacean species where sloughed skin can be collected.

Introduction

Various methods of DNA collection for cetaceans have been utilized in the past including biopsy darting, fecal sampling and sloughed skin collection. While biopsy sampling promotes an accurate match between observed whale identity and DNA collected, it is an invasive technique. Collection of fecal matter is non-invasive but often does not yield high quality DNA. In this study, we demonstrate that sloughed skin collection provides a non-invasive, low-cost means of acquiring high quality DNA for individual identification of humpback whales (*Megaptera novaeangliae*).

In the past, sloughed skin collection has been used to investigate group composition of humpback whales (e.g. Amos et. al. 1992, Valsecchi et. al. 1998). Valsecchi et al. demonstrated that the same number of genotypes and sex composition in pods was consistent with field observations. In only 1% of cases there were more genotypes than whales observed. In addition, multiple samples from a single collection event were gathered for 15 single whales. Every set of samples for each 'singleton' yielded a single genotype indicating that all skin fragments collected at that one site belonged to the same individual.

In our study, we demonstrate the accuracy of individual identification over time. We have genotyped samples from known whales across years to prove that sloughed skin gives identical, uncontaminated, genotypes over time. Reliance on genotypic data will prove valuable when answering questions about humpback whale ecology. Here, we emphasize the use of sloughed skin for individual identification to investigate reproductive ecology rather than population genetics. Eventually, with the integration of genetic data and photo identification of known whales and associated behavioral observations, we will have the opportunity to better evaluate reproductive success and assess aspects of reproductive biology that no one approach can address in isolation.

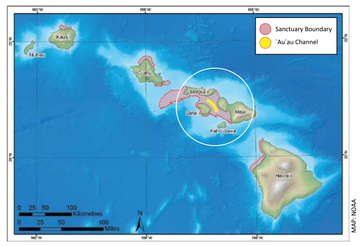

Figure 1. Sloughed skin samples were obtained within the *National Marine Sanctuary* in the waters of the *'Au'au Channel* between the four islands region of Maui, Lāna'i, Moloka'i and Kaho'olawe.

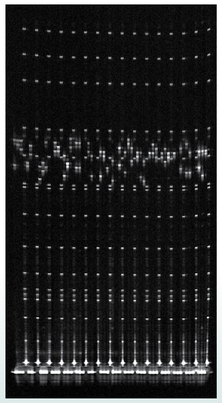

Figure 2. A computer image of a gel ran using a Li-Cor 4200 automated sequencer and M13 labels.

Methods

Data collection:

- Sloughed skin samples were collected as part of a long-term study of humpback whale behavior over the course of fourteen seasons (1995-2009, excluding 1997).
- Sloughed skin was taken from both above and below the water's surface with an aquarium net.
- Samples were collected from February through April in waters off the coast of Maui, Hawaii and were stored in salt since collection.

Sample distribution:

- DNA was extracted (using Qiagen DNeasy kits) from known individuals and any associated calves.
- Genotyping samples collected from the same individual at different sighting events allowed us to test the validity of the sloughed skin collection method.
- We genotyped a total of 49 samples, representing 15 individual whales.

Data analysis:

- 29 microsatellite loci were screened and 14 total loci were chosen for analysis.
- A MJ Research DNA engine (Waltham, MA) was used to perform PCRs in 10 µL volumes (10mM Tris-HCl, 50mM KCl, 2.0mM MgCl2, 0.2 mM each dNTP, 0.3 U Taq DNA polymerase, and 100 ng DNA template). PCR profiles involved one cycle at 94°C for 2 min; 30 cycles of 94°C for 30 s, annealing temperature for 30 s, and 72°C for 15 s; and one cycle at 72°C for 30 min.
- Products were visualized using M13 labels and Li-Cor 4200 and 4300 automated sequencers (Li-Cor Biosciences, Lincoln, NE).
- Genotypes were scored using SAGA v 3.3 (Li-Cor Biosciences, Lincoln, NE).
- GENEPOP version 3.4 (Raymond and Rousset 1995) was used to test for Hardy-Weinberg equilibrium. Exclusion probabilities were calculated using CERVUS.

Results

- Only 2 of 14 loci deviated significantly from Hardy-Weinberg equilibrium after Bonferroni correction.
- Probability of correctly identifying an individual based on the genotype produced with the chosen loci is 99.99999%.
- We obtained replicate samples for 12 individuals. In all cases, the individual genotypes were identical.
- In all but one case, known mothers shared one-half of their genotype with their observed calves. In the fifth case (F2 and offspring C3), there was a single-locus discrepancy requiring reanalysis.

Table 1. Descriptive statistics of the fourteen loci used in analysis. ** indicates significance after Bonferroni correction. n=sample size (calculated as part of a larger study).

Locus	n	Allelic range	Number of alleles	Ho	He	HW	GenBank accession no.
AC137	138	123-173	20	0.862	0.878	NS	DQ151647
GT129	139	103-115	5	0.496	0.500	NS	DQ151655
GATA028	133	111-205	8	0.474	0.458	NS	U93890
GATA098	123	96-134	11	0.634	0.817	**	U93892
CA234	134	198-222	12	0.955	0.846	NS	DQ151651
GATA417	138	208-248	12	0.870	0.889	NS	U93893
AC045	142	172-204	7	0.563	0.656	NS	DQ151645
GT575	139	156-195	14	0.835	0.817	NS	AF309698
CA141	139	191-207	9	0.540	0.693	**	DQ151659
GT271	130	114-140	10	0.708	0.806	NS	AF309694
GT310	140	121-125	3	0.186	0.172	NS	AF309696
GT195	142	175-187	8	0.683	0.724	NS	AF309692
GT023	113	128-140	7	0.708	0.710	NS	AF309690
CA232	136	165-197	14	0.779	0.854	NS	DQ151650

Table 2. Number of repeat samples of known individuals, years the individual was sampled and number of days they were sampled in years of multiple sampling dates. Identity is coded for F=female, C=calf (preceded by mother if known), and W=other whale (sex and age not verified).

Identity	Number of samples	Years sampled	Within season resampling
F1	7	2002, 2006, 2009	2006: 3 sampling dates
(F1) C1	6	2002	2002: 4 sampling dates
(F1) C2	1	2006	
F2	8	1995, 1996	1995: 3 sampling dates
(F2) C3*	5	1995, 1996	
F3	2	2006	
(F3) C4	1	2006	
F4	3	1995, 2008	
(F4) C5	1	1995	
C6	2	1996	
C7	3	2000	
W1	2	1999	
W2	4	1996	
W3	2	2002	
W4	2	1998	

*This individual was sighted in 1995 as a calf and again in 1996 as a yearling. Relationship to mother requires additional analysis.

Conclusions

- The tissue viability and genotyping success indicates that sloughed skin is a valuable tissue source for DNA extraction.
- The heterozygote deficiency in two of the fourteen loci indicates an overabundance of homozygotes in the population and can be attributed to various factors including the presence of null alleles, large allele drop out, or technical and scoring error.
- This unique combination of loci, along with the optimized protocol, will provide further insight for future analyses of humpback whale social dynamics, reproductive success, ecology and life history.
- Our ability to identify individuals with such high exclusion probabilities highlights the utility of the chosen microsatellite markers.
- Comparing genotypes from multiple samples of known individuals validates sloughed skin collection by proving that field observations of individuals have matched with genetic identification.
- This analysis only includes a subset of samples from a much larger dataset. The protocol that has been optimized in this study, including the validation of sloughed skin collection methods for individual identification, can now be applied to additional samples in order to gain a broader understanding of humpback whale reproductive ecology.
- Integrating long term photo identification and genetic analyses of known individuals with behavioral observations of known individuals will provide the tools necessary to generate paternity analyses and better understand how, and to what extent, different mating strategies influence reproductive success in humpback whales.

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