GENETIC IDENTIFICATION OF KEMP'S RIDLEY SEA TURTLE FROM EGGSHELLS FOUND AT A DEPREDATED NEST AT A NOVEL NESTING SITE

Cole McGough[1], Jenny Estes[2], Drew Hataway[1]
[1]Department of Biological and Environmental Sciences, Samford University
[2]Department of Biology, Chemistry, Mathematics & Computer Science,
The University of Montevallo
Correspondence: Drew Hataway rahatawa@samford.edu

ABSTRACT

Many sea turtles return to the same nesting grounds each year but due to factors such as climate change and anthropogenic development are forced to find new and novel nesting sites. Identifying sea turtle species using these novel sites is key to conservation. A depredated sea turtle nest was found at a novel nesting site in Alabama in the Summer of 2019. Based on the reproductive biology and natural history of turtle species inhabiting the region, this nest is most likely to have been one of four species known to inhabit the Northern Gulf of Mexico: Loggerhead, Kemp's ridley, or Green. Predation left limited evidence including relative size of the nest and eggshells devoid of any other tissues that could be used to determine turtle identification. Genomic DNA extraction from eggshells returned low yields due to limited amounts of DNA found within eggshells as well as the storage conditions of samples prior to extraction. Isolation required use of liquid nitrogen and an extended incubation in lysis buffer to maximize yield. A portion of the mitochondrial DNA was then amplified, and the turtle identified as a Kemp's ridley sea turtle (*Lepidochelys kempii*, Garman 1880).

INTRODUCTION

Sea turtles are known to exhibit natal homing for nesting beaches and there are increasing and emergent threats to nesting grounds (Bowen and Karl 2007, Lamont et al. 2023, Robinson et al. 2023), therefore determining nesting locations is vital to conservation efforts (Lamont et al. 2023, Scott et al. 2022). The Kemp's ridley (*Lepidochelys kempii*) is currently listed as critically endangered and their primary nesting site is located along the Gulf of Mexico (GOM) within 30.2 km of Rancho Nuevo area in Tamaulipas, Mexico (Bevan et al. 2016, Wibbels and Bevan 2019). Since the 1970's, a head start program has been in place to aid in species recovery and a second viable nesting location at Padre Island National Seashore in Texas, USA has been established. However, between 1989-2014, 118 Kemp's ridley nests were documented outside these primary nesting grounds (Shaver et al. 2016, Shaver and Caillouet 2015) suggesting potential for exploratory nesting similar to what has been seen in loggerhead sea turtles (*Caretta caretta*) in the Mediterranean (Hochscheid et al. 2022). Sea turtle rookeries are ephemeral over geologic time and breakdown of fidelity to natal homing grounds is required for new rookeries to be established perhaps in the face of *L. kempii* recovery (Johnson et al. 1999). It is imperative to monitor potential new nesting areas in the face of a changing climate, anthropogenic disturbance, and the complex population dynamics of this critically endangered species (Bevan et al. 2016, Wibbels and Bevan 2019).

Developing methodologies and strategies for how to recognize the presence of a rare and endangered species without direct visual confirmation of the mother or hatchlings is an important step in these monitoring efforts. With live specimens not always available for DNA extraction, alternative DNA sources have been used to identify species and haplotypes including bones (Krestoff et al. 2021), dead hatchlings or, eggshells collected within 15 hours of deposition with their contents discarded (Lamont et al. 2023, Shamblin, Dodd, Bagley, et al. 2011, Shamblin et al. 2012). Egg shells are expected to have lower DNA concentrations that tissues such as skin or blood (Schmaltz et al. 2006). Multiple studies have successfully used unincubated eggs for amplification of RFLPs, mtDNA, and microsatellites(Lamont et al. 2023, Moore et al. 2003, Shamblin, Dodd, Williams, et al. 2011). Shamblin et al. (2011) reported

unsuccessful amplification of DNA samples from swabs of the outer portion shells described in Schmaltz et al. (2006). This study aimed to (i) develop a method for successfully extracting template DNA from eggshells that had been opened and exposed for more than 15 hours and little to no associated membranes or other tissues and (ii) identify the species of turtle nesting at a novel site discovered by volunteers working with a local sea turtle conservation group.

METHODS

The eggs used in this study were found in a depredated nest near Cedar Point, Mobile County, Alabama in July 2019 (Figure 1). This location is within the northeast portion of the Mississippi Sound and part of the northern Gulf of Mexico. Volunteers with Share the Beach and Alabama Coastal Foundation found and collected the eggshell fragments from the nest site within 24 hours of depredation. After collection, the eggshells were stored in dry bags at -20℃ for 4 years and finally in 95% ethanol at -80℃.

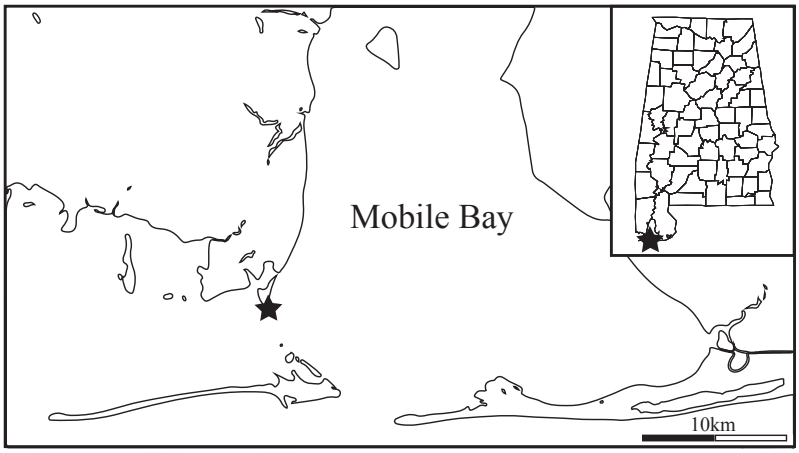

Figure 1: Location of depredated nest found in July 2019

A PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific) was used for extraction per the protocol with modification. The eggshells were flash frozen using liquid nitrogen, minced with a pestle and finally, incubated in lysis buffer for 24 h at 55℃. Extraction continued per the protocol following lysis. A positive control was created using loggerhead sea turtle tissue. All extractions were quantified using ThermoScientific NanoDrop Lite Spectrophotometer and TECAN infinite M200 PRO.

An amplicon from the mitochondrial NADH:ubiquinone oxidorecutase core subunit 4 (MT-NAD4) was generated for species identification. The 371 bp long MT-NAD4 amplicon was generated with the primers: F–5′AAGCTCATGTAGAAGCCCCA3′ and R–5′TGTTCGGCTGTGAGTTCGTT-3′ (Krestoff et al. 2021). PCR reactions were conducted in 26.5 µL volumes of 1.5 µL $MgCl_2$, 9.5 µL H2O, 12.5 µL Phusion TAQ, 1 µL F primer, 1 µL R primer, 1 µL template DNA under the following conditions: 2 min at 94˚C; 15 s at 95˚C, 1 min at 55˚C, and 20 s at 72˚C for 30 cycles; 10 min at 72˚C, and hold at 4˚C. Amplification was verified using a 1% agarose gel before sequencing. Alignment, trimming of primers and extraction of consensus sequences was done in Geneious Prime software v.24.0.3. Sequences were then compared to reference sequences within NCBI for species identification.

RESULTS AND DISCUSSION

Modifying the manufacturer protocol to include both a flash freezing step and an extended incubation provided the best results in relation to quantity and purity of DNA. We obtained a mean yield of 17.37 ng/µL and a mean purity of 1.639 (260/280). However, yield and purity data were not indicative of DNA quality and positive amplification was independent of samples demonstrating high yield or purity or both.

Additionally, sequence quality scores were typically low and required multiple rounds of resequencing. Sequencing and subsequent BLAST searches confirmed the species was Kemp's ridley sea turtle (*Lepidochelys kempii*).

The positive species identification using degraded eggshell tissue validates another technique for genetic identification of sea turtles and an additional tool for use in surveying novel nesting locations. This technique extends methodologies previously developed for fresh eggshells by Shamblin and others. Aerial and ground surveys have been used for such identification (Lamont et al. 2023; Scott et al. 2022). Ground truthing in these surveys included collection of tissues for genetic identification including undeveloped eggs, embryos, and/or dead hatchlings in some combination. This study provides additional methodologies for identification when those more DNA rich samples are not available due to predation events.

After four years of storage of a suboptimal tissue source, DNA was extracted and amplified for use in species identification. We recommend incorporating use of liquid nitrogen in eggshell processing as well as increased incubation times and temperatures as others have suggested (Shamblin, Dodd, Williams, et al. 2011). The larger conclusion and next steps are to engage nesting beach monitoring programs to ensure early and proper storage of any shells found. This could include providing these patrols with collection kits that include 50 mL conical tubes with 95% ethanol along with directions for storage in a freezer and a phone number of a research group to contact.

The results of this study also document a novel nesting site for a Kemp's ridley sea turtle in the bay system of Alabama. Kemp's ridley nests are periodically documented in low numbers along the coast of Alabama, but this is the first report of nest within the bay system (i.e. eastern portion of the Mississippi Sound).

DATA AVAILABILITY AND ETHICS STATEMENT

The sequence data presented in this study can be found in online repositories. The name of the repository and accession number are: https://www.ncbi.nlm.nih.gov/genbank, PP858890. Ethical review and approval were not required for the animal study as there were no materials acquired from living individuals. Egg shell samples were obtained postmortem (Alabama Coastal Foundations FWS permit 100012).

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