DEVELOPMENT OF A MINIMALLY DESTRUCTIVE METHOD OF ANCIENT DNA EXTRACTION FROM NARWHAL (MONODON MONOCEROS) TUSK MATERIAL

by

K.M.C. Smith
A Thesis
Submitted to the
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of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Environmental Science and Policy

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Date: Fall Semester 2019
George Mason University
Fairfax, VA
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DEDICATION

First, this is dedicated to my wonderful husband, Iain. Four years ago, we began our marriage almost a year after my brain surgery complications and enrolled in graduate school together. What on earth were we thinking? Life has definitely thrown us some curveballs along the way, but I thank God every day for the life we have together and the warrior He has given me. You were always there encouraging me to pursue my passion, even when I struggled with balancing brain surgery recovery, a graduate program, and going back to work. Second, this is also dedicated to my family, both the old and the new. I would like to thank both the Couches and the Smiths for your encouragement as I worked my way through this program. You know it has been a long and hard process, and you never once doubted I would finish. Finally, I dedicate this to my father, Dr. Mark Couch, whose “pony joke” has forever colored my outlook on life. According to the “pony joke,” a family, worried their son was too optimistic, took him to see a psychiatrist to dampen his optimism. The psychiatrist placed the boy in a room piled high with horse manure. The boy jumped into the pile of manure in excitement and began digging furiously. Expecting the boy to recoil in disgust, the baffled psychiatrist exclaimed in shock, “What are doing?” The boy paused and said beaming, “With all of this manure around, there must be a pony in here somewhere!” Dad, this is my pony…or, in this case, my unicorn.
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LIST OF ABBREVIATIONS AND SYMBOLS

Base pair ........................................................................................................... bp
Before Common Era ...................................................................................... BCE
Bovine serum albumin .................................................................................... BSA
Centimeters ..................................................................................................... cm
Common Era .................................................................................................... CE
Convention on International Trade in Endangered Species ...................... CITES
Deoxyribonucleic acid ............................................................................... DNA
Dichlorodiphenyltrichloroethane ............................................................... DDT
Diethyl pyrocarbonate ............................................................................... DEPC
Ethidium bromide ....................................................................................... EtBr
Gram .................................................................................................................. g
Hertz .................................................................................................................. Hz
International Union for Conservation of Nature and Natural Resources ........ IUCN
International Whaling Commission ......................................................... IWC
Kilograms ........................................................................................................ kg
Kiloherertz ......................................................................................................... kHz
Kilometers ......................................................................................................... km
Magnesium chloride ..................................................................................... MgCl₂
Marine Mammal Protection Act ................................................................ MMPA
Meters .............................................................................................................. m
Microliter ......................................................................................................... μL
Milligram ......................................................................................................... mg
Milliliter ........................................................................................................... mL
Millimeters ....................................................................................................... mm
Millimolar ......................................................................................................... mM
Mitochondrial DNA ....................................................................................... mtDNA
Molar ............................................................................................................... M
Nanograms per gram .................................................................................... ng/g
National Center for Biotechnology Information ........................................ NCBI
Nuclear mitochondrial DNA .................................................................... numt
Polychlorinated biphenyl .............................................................................. PCB
Polymerase chain reaction .......................................................................... PCR
Revolutions per minute ................................................................................. rpm
Total allowable harvest ............................................................................... TAH
Unified atomic mass unit .............................................................................. U
ABSTRACT

DEVELOPMENT OF A MINIMALLY DESTRUCTIVE METHOD OF ANCIENT DNA EXTRACTION FROM NARWHAL (MONODON MONOCEROS) TUSK MATERIAL

K.M.C. Smith, M.S.
George Mason University, 2019
Thesis Director: Dr. Lorelei Crerar

Narwhals (Monodon monoceros) are elusive animals, and have inspired myths, legends, and awe for centuries, which is exacerbated by their inaccessibility in the heavily iced high Canadian Arctic waters. With a current “Near Threatened” classification by the International Union for Conservation of Nature and Natural Resources (IUCN) and a total population estimate of roughly 80,000 animals, unlocking the genetic code of this species may be the key to beginning to unravel much of the mystery that surrounds them, both in the past and the future. This study compared a drilling method and a grinding method to provide a reliable, minimally destructive method to extract ancient narwhal DNA from samples that need to remain physically undamaged for display purposes. Short pieces of unprocessed pre-1972 narwhal tusk (n = 50) were obtained from Pond Inlet, Nunavet Canada. This study utilized narwhal cytochrome b mitochondrial DNA (mtDNA) data from the National Center for Biotechnology Information’s (NCBI) GenBank, where two
primers, NAR-4 (581 bp) and NAR-6 (241 bp), were created for use in Polymerase Chain Reaction (PCR). Using a grinding technique on the tusk surface, the study outlines a reliable method to extract ancient deoxyribonucleic acid (DNA) from narwhal tusk and amplify it for further analysis using PCR. The amplified DNA from the grinding method was compared to the traditional drilling method using electrophoresis and the grinding method yields the same level of amplification of DNA as the drilling method. The extracted DNA was then sequenced using the designed primers and compared to narwhal mitochondrial DNA samples in GenBank to positively confirm narwhal as the sample’s identity. This study’s grinding technique caused a significant reduction in physical marring to the surface of the narwhal tusk samples and provided evidence for a reliable method to extract ancient narwhal DNA while preserving historical samples for undamaged display.
CHAPTER ONE: INTRODUCTION

Narwhals (*Monodon monoceros*) are elusive animals, but they have inspired awe and legends in human communities for centuries. Historically, the narwhal and its iconic tusk inspired many myths. This chapter reviews some of the myths and legends about unicorns and their infamous tusks. Additionally, it also reviews the current scientific information concerning their biological characteristics, habitat, and behavior, in particular the unique adaptations possessed by narwhals that allow them to survive in a harsh Arctic sea ice environment. Lastly, threats that influence the current populations of narwhals will be discussed – in order to better understand how to protect these populations.

Multiple threats, such as unsustainable hunting, industrial development, and Arctic climate change, could heavily impact the persistence and future survival of narwhals.

Whilst their scientific name, *Monodon monoceros*, means “one tooth, one horn,” the name “narwhal” originates from two Old Norse words; the prefix “nar” meaning corpse and the word “vhal” meaning whale (McLeish, 2013). These “corpse whales,” were so-called because their coloring looked very similar to waterlogged corpses found at sea, and it was believed that eating their flesh made seafaring people fatally ill (Lopez, 1985; Larson, 1917).

Taxonomically, narwhals belong to order Cetartiodactyla, which contains even hoofed ungulates and cetaceans, infraorder Cetacea, parvorder Odontoceti, or toothed
whales (Rosel et al. 2017). Adult narwhals are generally a darker gray or black color along their dorsal side, with dappled whites and creams along their ventral side. However, juvenile narwhals tend to be more of a bluish-gray color from the time they are born and gradually become more dappled with whites and creams as they age. As narwhals age, their coloring changes, with their dappling becoming more pronounced, until they are almost completely white (Reeves & Tracey, 1980).

Narwhals, like their taxonomic “cousin,” the beluga (Delphinapterus leucas), have a rounded head with a large melon; however, narwhals do not possess the lipped beak of belugas. Narwhals have no dorsal fin, but rather a small ridge that runs along their back. Their flippers are also smaller than would be expected for an animal of its size (Reeves & Tracey, 1980). Narwhals are sexually dimorphic, with the males reaching an average length of six meters (m) and weighing roughly 1600 kilograms (kg) and the females reaching an average length of 5m and weighing roughly 850kg (Harington, 2008). Their lifespan is estimated to be equivalent to that of belugas, which is somewhere between 50 years and 80 years, however specimens as old as 100 years have been estimated (Garde et al. 2015).

Narwhals are born with two tusks, or teeth, that may erupt. The tusk remains imbedded in the maxillary bone, and generally, only the left tusk erupts horizontally through their upper lip (Nweeia et al. 2008). The erupted tusk always spirals in a counterclockwise direction (Reeves & Tracey, 1980). Tusks are predominately found in males and can grow up to 3m in length, however they are occasionally also found in females (Reeves & Tracey, 1980). A rare occurrence of double tusks is found in roughly
1.5% of narwhals, and in this instance, the second right tusk grows to almost the same length as the left tusk (Nweeia et al. 2008).

Martin Nweeia, a dentist, studied the narwhal tusk in detail to determine its composition. The hardest material of the tusk surrounds the pulp and the outermost layers of the tusk are a softer, more flexible material (Nweeia et al. 2008). Unfortunately, narwhal tusks are susceptible to breaking or snapping near the tips. Broken tusks in older narwhals are a common occurrence and exposing the dental pulp of the tooth to the environment would be extremely painful. Many broken tusks have been observed with a “filling” and native Inuit people have even observed a younger male inserting the point of his tusk into the older male’s broken tusk (Reeves & Tracey, 1980). The younger males appear to break off the very tip of their own tusks to fill these broken tusks (Reeves & Tracey, 1980). With the extreme pain that results from the exposure of the dental pulp to the environment might cause, the evolution of a behavioral system where young males might altruistically “plug” the tooth cavity of older, more dominant whales might make sense, especially if aiding the older male potentially leads to the younger male gaining status or benefits in the narwhal social structure.

Due to the sensitivity of the tusk, Nweeia et al. (2008) suggested that the narwhal’s tusk perhaps served as a sensory organ to determine temperature, salinity, and other chemical factors of its surroundings. Since a thorough examination of the narwhal tusk had never been conducted before, the hypothesis that the tusk was a sensory organ quickly gained popularity with the media as the “true” purpose of the tusk. Leading agencies, such as National Geographic, even began passing this single examination off as
fact, despite any further supporting evidence (Lee, 2014). However, if the tusk were solely a sensory organ, one would expect such a characteristic to be present in both sexes, rather than solely in males. Although further research clarified the composition of a narwhal’s tusk, the tusk probably has other functions aside from sensing the environment (Nweeia et al. 2008).

Several other hypotheses as to the purpose of the narwhal’s tusk have been suggested. Some believe the tusk is used as a rake along the bottom of the ocean or as a tool for finding food (Reeves & Tracey, 1980). Others believe that it helps the narwhal break through the ice to breathe or to fend off attacks from polar bears (Nweeia et al. 2008). The most likely purpose of the tusk is that it serves as a secondary sexual characteristic, visually allowing the females to assess the fitness of the male (Reeves & Tracey, 1980). Male narwhals have been observed “jousting” with other males when competing for a female’s attention, making the use of the tusk as a secondary sexual characteristic, a more widely accepted hypothesis amongst marine mammologists (Nweeia et al. 2008).

**Habitat**

Narwhals are found primarily in the cold waters of the Arctic Ocean between 70°N and 80°N (Reeves & Tracey, 1980). They appear to prefer to spend a majority of their time in locations with temperatures ranging between -1.2°C and -1.6°C (Laidre et al. 2003). Most narwhal populations are concentrated in Baffin Bay and Davis Strait in Western Greenland, with an estimated population of roughly 45,000 to 50,000 narwhals.
in this region (Laidre & Heide-Jørgensen, 2011). Some narwhal populations have been sighted in the eastern North Atlantic off of Svalbard, Norway, but the extent of their numbers in this region is unknown (Lydersen et al. 2007). However, unlike most other cetaceans, narwhals do not spend most of their time in open water, but rather are found predominately in thick pack ice, utilizing leads and cracks to breathe (Laidre & Heide-Jørgensen, 2011).

Narwhals migrate, moving from summering grounds in the high Arctic to their wintering grounds in the lower Arctic near Western Greenland (Laidre et al. 2004). These large populations of narwhals in Western Greenland spend their summers around Somerset Island and then begin their migration in September to their wintering grounds in Baffin Bay (Heide-Jørgensen et al. 2003). Baffin Bay is comprised of the thick pack ice during the winter; therefore, narwhals that feed in this area rely on lead and crack openings in the ice to breathe (Heide-Jørgensen et al. 2003). Tagged narwhals in the Baffin Bay populations appear to express high site fidelity, often returning to the same summering and wintering grounds each year. These populations also follow the same paths of migration year after year, where changes in the weather signal the beginning and end of their migration (Heide-Jørgensen et al. 2003).

Narwhals possess several adaptations that aid them in their icy habitats. They have a thick layer of blubber, averaging 70 to 80 millimeters (mm) thick, helping to insulate them from the cold temperatures of the water (Reeves & Tracey, 1980). Narwhals average 25 minutes between breaths, spending 15 minutes of that interval transitioning between layers in the water column and only 10 minutes foraging for food.
(Laidre et al. 2003). They can dive to over 1.5 kilometers (km) on a single breath (Heide-Jørgensen et al. 2002). Their breath-holding and deep-diving adaptations allow narwhals to thrive in areas with heavy pack ice.

Another unique adaptation of the narwhal is the structure of their muscles. Researchers have found that narwhals’ muscles are composed of both slow twitch muscle fibers and fast twitch muscle fibers; however slow twitch muscle fibers comprise roughly 80% of their muscle tissue (Williams et al. 2011). This high percentage of slow twitch muscle fibers suggests that narwhals are best adapted for slower, long distance swimming, rather than faster swimming (Williams et al. 2011). Narwhals have some of the highest concentrations of myoglobin recorded among all cetaceans, storing most of the oxygen available to them on dives primarily in muscle tissue, followed by blood-bound oxygen (Williams et al. 2011).

Due to the extreme conditions that narwhals live in most of the year and their high site fidelity in logistically difficult to access locations, studying their movements is often challenging. Jostling in heavily iced waters means that satellite tags often malfunction or fall off, making data collection inconsistent (Laidre et al. 2003). Inaccessibility also means that obtaining tissue samples from narwhals is often limited to samples taken from subsistence hunts, or from entrapments (Williams et al. 2011). The compilation of these factors indicates that obtaining data on narwhal ecology can be a challenging endeavor.
Behavior

Narwhals, like many cetaceans, are highly social. They are found in pods of up to 21 animals, and pods typically consist of three to eight animals (Reeves & Tracey, 1980). Pods can be composed of a single sex, or mixed sexes (Reeves & Tracey, 1980). Narwhals communicate using three distinct kinds of vocalizations: echolocation clicks, reaching maximum frequencies of 160 kilohertz (kHz), tonal-pulsed signals with frequencies ranging between 500 hertz (Hz) and 24kHz, and whistles with frequencies ranging between 300Hz and 18kHz (Shapiro, 2006). The clicks are used for echolocation and the whistles presumably have a communication function (Marcoux, 2008). The sounds they produce have often been described as sounds similar to “growls” or “groans”, much like that of a “bear” or “cow” (Reeves & Tracey, 1980).

Female narwhals become sexually mature around the age of eight or nine-years-old and are expected to carry their first pregnancy between the age of nine and 10-years-old (Garde et al. 2015). Less is known about the age at which male narwhals reach sexual maturity, but it is estimated that they become mature between the ages of 12 and 20 years (Garde et al. 2015). Based on these estimates, narwhals should theoretically breed every two to three years. Female narwhals do appear to go through menopause like other cetaceans, such as killer whales (*Orcinus orca*) and pilot whales (genus *Globicephala*) (Ellis et al. 2018). Researchers have estimated that this makes their average generation time roughly 30 years (Garde et al. 2015).
Narwhal mating behavior has never been observed in the wild, and estimates are provided based on data collected from Inuit hunts and the mating behaviors of their closest taxonomic relatives, belugas. Narwhals are believed to begin mating in late spring and mating occurs exclusively within local populations of several pods (Heide-Jørgensen & Garde, 2011). They have an estimated gestation period of roughly 14 months and give birth to their young between May and June (Heide-Jørgensen & Garde, 2011). Newborn narwhal calves are estimated to be 160 centimeters (cm) in length and roughly 80kg in weight. Calves suckle for approximately 20 months, an estimation based again on their beluga relatives (Reeves & Tracey, 1980). Narwhal calves are easily identifiable, as they are a darker gray color than their parents, but they are often mistaken as beluga calves (Heide-Jørgensen & Garde, 2011).

Unlike belugas, attempts to maintain narwhals in captivity have been unsuccessful. In the 1970s, the Vancouver Aquarium attempted to institute a breeding program for narwhals in their facilities. They captured two females, three calves, and purchased one male (Reeves & Tracey, 1980). However, all the narwhals died within four months from infections or pneumonia (Newman, 1994). The slightly warmer water temperatures in the aquarium were proposed as the underlying cause of the pneumonia deaths (Newman, 1994). Around the same time, the New York Aquarium also attempted to maintain a captive young narwhal calf, but this calf died within one month of living at the aquarium (Reeves & Tracey, 1980). No further efforts have been attempted to keep narwhals in captivity.
Narwhals feed primarily on Greenland halibut (*Rheinhardtius hippoglossoides*), but also consume polar cod (*Boreogadus saida*) and cephalopods (Reeves & Tracey, 1980). However, their diets change throughout the year, including how much food they consume. During the summer months, narwhals are observed to consume small amounts of polar cod, Arctic cod (*Arctogadus glacialis*), and cephalopods (Laidre & Heide-Jørgensen, 2005b). During the winter months, narwhals undergo heavy feeding on Greenland halibut (Laidre & Heide-Jørgensen, 2005b). Different narwhal populations can exhibit different diving behaviors for their prey, with some populations diving deeper, and others diving shallower for their prey (Laidre et al. 2003).

Narwhals are subject to predation by other species, such as polar bears (*Ursus maritimus*), killer whales, and humans. The biggest predation threat that narwhals face, however, comes from human hunting (Reeves & Tracey, 1980). Narwhals are susceptible to entrapments in the sea ice, called “sassats” in the native language of the Inuit, which makes them vulnerable to predation by humans and polar bears (Smith & Sjare, 1990). Killer whales also target narwhals while they inhabit their summering grounds (Williams et al. 2011). When targeted by killer whales, narwhals submerge for prolonged periods or swim farther into areas with pack ice (Williams et al. 2011). They also engage in slower and quieter movements when evading killer whales, as well as maintaining a tight-knit group in shallower waters (Laidre et al. 2006).
Conservation

Narwhals are classified as “Least Concern” by the International Union for Conservation of Nature and Natural Resources (IUCN) (Lowry et al. 2017). However, due to insufficient data, they were only recently listed in 2008, and were considered to be “data deficient” prior to this (Lowry, Laidre, & Reeves, 2017). As noted above, narwhals are elusive, logistically difficult to access, and spend much of their time in the high Arctic waters and/or under ice, making population estimates difficult to obtain. Narwhal populations in the high Canadian Arctic waters were estimated to be roughly 45,000 animals (Innes et al. 2002). The estimated global population of narwhals is estimated to be roughly 80,000 animals, but the overall trend concerning how the global population is doing is unclear due to insufficient data (Lowry et al. 2017).

Narwhals are listed under the Convention on International Trade in Endangered Species (CITES) of Wild Flora and Fauna Appendix II and under the Convention on the Conservation of Migratory Species Appendix II (Lowry et al. 2017). This restricts international trade in narwhal products. While narwhals are listed under the United States’ Marine Mammal Protection Act (MMPA) of 1972, which restricts hunting to subsistence only, given that most narwhal populations are found in the high Arctic, Canadian and Greenland legislation may be more relevant to their protection (Marine Mammal Protection Act, 1972). The International Whaling Commission (IWC) has discussed the status of narwhals (NAMMCO, 2018). However, IWC deliberations about protecting narwhals may be rather moot considering that Canada withdrew from the IWC.
in 1981 and Greenland opposes the IWC’s policy debates concerning aboriginal subsistence whaling of narwhals (The New York Times, 1981; Whale and Dolphin Conservation Society, 2004; Caulfield, 1993). Moreover, trade of narwhal products can be more heavily regulated in different countries; for example, the European Union has banned importation of any narwhal tusks or products (Lowry et al. 2017). Despite these efforts to protect remaining narwhal populations, they continue to face increased threats from commercial industry developments, climate change, and hunting.

**Commercial Industries**

With the warming of the Arctic waters, sea passages are remaining open for longer periods of time, and several countries, most prominently the United States and Russia, have proposed oil and gas developments in the Arctic (Gautier et al. 2009). It is estimated that roughly 30% of the world’s gas and 13% of the world’s oil may be present on these Arctic shelves, further driving an interest in future developments (Gautier et al. 2009). Many of the potential oil and gas reserves are specifically concentrated in the Arctic near Russia and western Greenland. This has, unfortunately, prompted an increase in the number of seismic surveys, often with an associated production of high intensity noise, to determine the exact amounts of oil and gas present at these locations (Gautier et al. 2009).

Although the presence of icebergs and the constant formation of sea ice provides a high level of background noise in the Arctic waters, narwhals primarily rely on acoustic communication to alert them to various events underwater, as well as to communicate
with other members in their pods (Heide-Jørgensen et al. 2013). Narwhals have exhibited elusive and skittish behavior when presented with louder sounds underwater, usually fleeing to denser pack ice (Laidre et al. 2006). Seismic testing for oil and gas reserves exposes narwhals to louder underwater sounds. With increases in seismic testing, increased noise pollution could be contributing to elevated instances of entrapment events in narwhal populations (Heide-Jørgensen et al. 2013).

The recent openings of offshore Greenland halibut fisheries operations are another cause for concern of the narwhals’ survival. Since the 1980s, Greenland halibut catch numbers have increased substantially (Jørgensen et al. 2014). These fisheries are targeting the Greenland halibut species found around 1000 m in depth, which is the same depth for many feeding West Greenland wintering narwhal populations (Jørgensen et al. 2014). There is concern that these fisheries could potentially damage the Baffin Bay Greenland halibut stocks, ultimately resulting in a smaller prey stock for the narwhals that winter there (Laidre & Heide-Jørgensen, 2005b). Although health concerns for narwhals – resulting from injury or entanglement in fishing gear – is an issue in the Arctic, incidents may often go unreported due to the lack of monitoring in high-density areas (Kühn et al. 2015; NAMMCO, 2018).

With all the commercial industry developments that are beginning to appear in the high Arctic areas, pollution has also become an issue. Since anthropogenic contaminants biomagnify throughout the food chain, higher levels of toxins in narwhals’ prey species, such as the Greenland halibut, can become an issue for narwhals’ health. Toxic chemicals such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs)
have been found accumulated in narwhal blubber (Muir et al. 1992). For example, narwhals in Svalbard, Norway, averaged a PCB sum of roughly 9900 nanograms per gram (ng/g) lipid, and DDT was the predominant pesticide found in narwhal blubber; the PCB and DDT levels found in narwhals were 10 to 30 times higher than levels found in other high Arctic mammals, such as polar bears and harp seals (*Pagophilus groenlandicus*) (Wolkers et al. 2006). Highest concentrations of heavy metals, such as mercury and cadmium, are often found in the muscles, liver, and kidneys, rather than the blubber – the latter being where organochlorine pollutants tend to accumulate (Wagemann et al. 1983). The high levels of pollutants present in narwhal blubber and tissues are of concern for indigenous hunters that rely on subsistence hunting and could potentially result in toxic pollution in those communities that rely on narwhals for food.

**Climate Change**

The biggest current threat facing narwhals is climate change, more specifically the loss of increasingly more Arctic ice each year (Laidre & Heide-Jørgensen, 2005a). From 1979 through 2012, annual Arctic sea ice cover declined roughly 3.5% to 4.1% per decade, with the highest rates occurring in the perennial ice, which declined roughly 9.4% to 13.6% per decade (Intergovernmental Panel on Climate Change, 2013). In addition to melting Arctic ice, changes in the ocean’s salinity, temperature, and thermohaline circulation are also causes for concern (Laidre & Heide-Jørgensen, 2005a). As melting ice increases, the salinity of the Arctic waters decreases, allowing very fresh surface water to be easily refrozen (Laidre & Heide-Jørgensen, 2005a). Higher carbon
dioxide levels from anthropogenic activities are prompting rapid climate warming, which ultimately contributes to the melting of Arctic ice (Intergovernmental Panel on Climate Change, 2007). These oceanic issues, such as temperature and salinity, could impact the Arctic marine ecosystems, causing shifts in zooplankton blooms or feeding grounds (Laidre & Heide-Jørgensen, 2005a).

The impact warmer global temperatures have on the Arctic marine ecosystems has been severely under-evaluated in comparison to the Antarctic (Wassmann et al. 2011). Overall, the annual sea ice coverage in the Arctic has decreased by about 3% over the last 30 years, with more recent estimates placing that number closer to 12.8% per decade since 1981 (Laidre & Heide-Jørgensen, 2005a; Intergovernmental Panel on Climate Change, 2019). Despite this collective decrease in annual sea ice coverage in the Arctic, areas in the Canadian high Arctic, Baffin Bay, and West Greenland have had increased sea ice coverage over the last 30 years, possibly due to higher freshwater inputs resulting in easier freezing conditions (Laidre & Heide-Jørgensen, 2005a).

Narwhals face many risks stemming from the percentage reduction in sea ice coverage. Since narwhals express high site fidelity and return to the same summer and wintering grounds each year, they are one of the species most at risk if they completely lose their pack ice coverage. They feed primarily during the winter, where they seem to prefer concentrating in areas with high levels of pack ice (Laidre & Heide-Jørgensen, 2011). With the Arctic ice continuing to melt, and the area of such high pack ice diminishes, narwhals will be potentially more exposed to their predators, like the polar bears which are also restricted to diminishing areas of sea ice and are locally increasing.
as a result. Narwhals also face higher risks of hunting from the indigenous Arctic communities, as well as loss of prey species as more fisheries and commercial industry developments in ice-free Arctic waters open (Laidre & Heide-Jørgensen, 2005b).

Many other cetaceans, species such as gray whales (*Eschrichtius robustus*) and killer whales, appear to be using the melting ice as an opportunity to expand their range farther north into the Arctic (Moore & Huntington, 2008). Unfortunately, narwhals are highly adapted to fill a specific hunting niche as a predator, and the presence of more cetaceans could create competition between species for specific prey. Moreover, narwhals facing increased predation from killer whales, may be poorly adapted to adequately escape due to their physiological adaptations toward slower swimming speeds (Williams et al. 2011). As water temperatures continue to increase, Arctic zooplankton and phytoplankton blooms could begin blooming earlier, causing a massive re-structuring of the marine ecosystem (Laidre & Heide-Jørgensen, 2005a). This could drastically disrupt the narwhals’ migration and feeding patterns, which occur at roughly the same time every year (Heide-Jørgensen et al. 2003).

With narwhals being so rigid in their migration patterns, they may be poorly adapted to adjust to rapid changes in climate. They spend a majority of their time in areas with heavy pack ice and, naturally, entrapments become a very real possibility. An entrapment occurs when the weather changes abruptly, causing fast ice to form in a matter of a few hours, during which narwhals swim into areas that once contained an opening to the sea, only to be cut off from open water a few hours later (Laidre et al.
2012). Some entrapments disappear almost as quickly as they developed, allowing animals to escape, whereas others become lethal for the narwhals.

The indigenous hunters are often the first to notice these entrapments and often alert the authorities (Laidre et al. 2012). If the ice shows no sign of melting and re-opening, the authorities rule that the entrapment will most likely be lethal to the narwhals (Laidre et al. 2012). Once the entrapment is ruled as lethal to the animals, hunters are allowed to take narwhals for subsistence purposes; the animals they harvest do not appear to count against their annual quotas (Heide-Jørgensen et al. 2002). Due to recent changes in the climate, the beginning of the autumn freeze that signals the start of narwhal migration, has been occurring later. It is believed that with these delayed freezing times narwhals are more at risk for experiencing entrapment events (Laidre et al. 2012).

**Hunting**

The Norse, historically farmers and sea-faring people, began exploration and colonization of other countries in the early 700s Common Era (CE). Norse settlers had colonized south Greenland under the leadership of Erik the Red by 982 CE (McLeish, 2013). Due to the Norse’s reputation as talented sea-faring people, they began hunting whales long before other Europeans. Ohthere, a merchant possibly from the Tromsø area in northern Norway who was documented visiting the court of King Alfred the Great in approximately 890 CE, described visiting the indigenous Sami communities in northern Norway and the coast of the White Sea to hunt “horse whales” or walruses (*Odobenus rosmarus*) (The Alfred Committee, 1852). He mentions that “in his own country, is the
best whale hunting” with animals from 48 to 50 “ells” or 96 feet to 100 feet, however this seems improbable (The Alfred Committee, 1852). Whales hunted in this latitude, and with the technology available, were more likely to be bowhead whales (*Balaena mysticetus*) or minke whales (*Balaenopera acutorostrata*), but no mention was made of hunting narwhals.

In the thirteenth century, *Konungs skuggsjá – Speculum Regalae – The King’s Mirror*, written during King Håkon IV’s reign, described the different kinds of whales that had been discovered and hunted by the Norse while on their journeys. In this manuscript, the Norse outline the hunting of various whales; however, narwhals were excluded from hunting because it was believed that their flesh made men sick, which ultimately resulted in death, and earned narwhals the name of “corpse whale” (Larson, 1917). As the Norse settlers colonized further into Greenland, many of them traded with the indigenous Inuit populations for narwhal tusks. The Norse then further traded these tusks to the European markets (Pluskowski, 2004). Merchants typically sold any acquired narwhal tusks to royal dignitaries or other wealthy individuals. Thus, narwhal tusk became a symbol of prestige and wealth during the Middle Ages, which also supposedly protected their patrons from harm from hidden poisons (Pluskowski, 2004). Commercial whaling of Arctic species, especially the narwhal, began to increase in the 1700s. While European whalers did use some parts of the whale, narwhals were most often hunted for their ivory tusks and oil, which, when burnt, was less smoky than some other oils (McLeish, 2013). The myth of the unicorn had somewhat diminished at this point, but narwhal tusks were still considered a valuable commodity in society.
The Norse settlers eventually disappeared from their Greenland colonies in the 1400s, possibly due to the onset of the “Little Ice Age” and an associated change in climate (Fagan, 2000). At this time, the Inuit people began moving back into the area previously colonized by the Norse, and the hunting of narwhals increased (McLeish, 2013). With the onset of a harsher climate, where growing anything became extremely difficult, the Inuit people hunted narwhals. They used every part of the narwhal and often used narwhal tusks for tent poles or harpoon shafts (McLeish, 2013). Narwhal hunting activities are a part of the Greenland Inuit people’s way of life. The Inuit primarily hunt narwhals for subsistence, valuing their “muktuk,” composed of whale skin and blubber, for its high levels of vitamin C (Lee & Wenzel, 2004). Greenland currently allows a quota of 85 narwhals taken for subsistence hunting per year and has guidelines for the hunting of narwhals, such as specific hunting permit requirements and specific hunting seasons (Nielsen & Meilby, 2013). In Western Greenland, the Inuit communities maintain that hunting narwhals should be done as it was historically done, with kayaks, harpoons, and animal skin floats to prevent the animals from sinking (McLeish, 2013).

However, the Canadian government has no real restrictions on how narwhals are to be hunted, but they do have restrictions on the numbers or narwhals harvested by the Inuit communities (McLeish, 2013). In 1971, Canada imposed a quota of five narwhals per Inuit hunter per year (Armitage, 2005). A total allowable harvest (TAH) is pending acceptance and once it is approved, it will replace the current quotas, but until then, community quotas currently allow for the TAH of 704 narwhals per year (Shadbolt et al. 2015). Narwhals are primarily taken from four communities, as seen in the figure below:
1) Pond Inlet, 2) Clyde River, 3) Grise Fjord, and 4) Arctic Bay, where these communities can harvest as many as 1500 narwhals per year combined (Mansfield et al. 1975).

There are concerns that such high quotas potentially encourage the hunting of narwhals for their valuable tusks rather than for subsistence (Mansfield et al. 1975). Unfortunately, many narwhals sink after being shot and are never recovered due to the fact that “stuck and lost” whales are rarely recorded, ultimately reducing the total
reported numbers of narwhals killed per year (Mansfield et al. 1975).

Since narwhals are highly migratory species, and as noted previously, logistically inaccessible, it is difficult to obtain data on their population numbers. The best estimates of the impact of subsistence hunting on current narwhal populations are often obtained through the number of narwhals taken in each Inuit community (Neilsen & Meilby, 2013). Due to reductions in catches, a population decline has been implied in many communities. However, increased protection of narwhals has been met with resistance from some of the hunters. Many hunters are skeptical of government-imposed regulations and quotas, expressing concern that these regulations and quotas will negatively impact their way of life without improving the overall narwhal stock numbers (Nielsen & Meilby, 2013). However, in cases of entrapment, or “sassats,” Inuit hunters are often the first to kill the trapped narwhals (McLeish, 2013). Whether the decline in population numbers is due to overhunting or climate change is a controversial topic of debate (Nielsen & Meilby, 2013).

Myths and Legends

Unicorns

The notable feature of mature narwhals is their tusk, a highly valued item in the Middle Ages because of its mistaken identity as “unicorn horn.” The earliest record of the unicorn came from a Greek physician named Ctesias’ writings in the fifth century Before Common Era (BCE) (Lopez, 1985). He documented an equine-like creature from India that possessed a single horn on its forehead (Lopez, 1985). British scholar, Odell
Shepard, determined that the creature described by Ctesias was most likely a rhinoceros (McLeish, 2013). However, this description of the equine-like unicorn persisted through other writings. The writings of Aristotle, Pliny, and Isidore of Seville shared similar descriptions and served to only fuel the credibility of Ctesias’ original description of the unicorn (Lopez, 1985). A Roman named Claudius Aelianus, or Aelian, published his work *De natura animalium*, in which he outlined the description of a mythical unicorn called Cartazonus (Hekster, 2002). Like Ctesias’ earlier writings, Aelian’s Cartazonus is also described to resemble a horse-like creature from India, where the black horn of the unicorn spiraled in shape that ultimately culminated into a sharp point (McLeish, 2013). It has been suggested that these descriptions might be referring to a single-horned ungulate species, the oryx, which has a slightly cured horn that looks like it is spiraled (Waters, 2013; Draycott, 2015). This description gave rise to the common illustration of the unicorn horn.

Albertus Magnus, a German Catholic friar of the Dominican order, who was also known as Saint Albert the Great or Albert of Cologne – sought to outline a system of universal sciences for his Dominican brothers in his works *Physica* and *De animalibus*, a published concept of the “sea unicorn” was proposed (Collins, 2010; Miteva, 2017). He described a fish-like creature in the northern seas that also possessed a single horn in its forehead, much like the previous descriptions of unicorns, and he ultimately published this description of the “sea unicorn” in his work *De animalibus* (Pluskowski, 2004). Albertus Magnus’ observations of this “sea unicorn,” merged the descriptions of Claudius Aelianus’ unicorn horn shape with his descriptions of the fish-like creature.
Unicorns and Religion

Despite Albertus Magnus’ observations of what was most likely a narwhal, the equine-like picture of the unicorn persisted in part because of a translation mistake in the Bible. When translating the Hebrew word “re’em” for a horned animal found in the Old Testament, the Latin word, “monoceros,” was chosen and as the translation progressed from Latin into English, the word became “unicorn,” a name that unfortunately stuck (McLeish, 2013). With all of the religious upheaval during the Middle Ages, the Roman Catholic Church incorporated the unicorn into religious dogma, rather than admit there had been a mistake. This acceptance was further solidified with the appearance of narwhal tusk, marketed as “unicorn horn” to unsuspecting buyers, during the Middle Ages (Lopez, 1985).

Unicorns, thus, became synonymous with the Virgin Mary and Jesus Christ in Christianity. Through the writings of St. Eustace of Antioch and Bishop Ambrose of Milan, unicorns were depicted as extremely strong and fearsome beasts (Pluskowski, 2004). This allowed unicorns to be portrayed as fierce saviors and avengers of evil in medieval art, usually depicted slaying an evil beast with its mighty horn. The “Unicorn Tapestries,” woven in the early 16th century CE, are an example of this religious symbolism, symbolically linking the unicorn with Jesus Christ (McLeish, 2013). Due to the indisputable acceptance of the unicorn into the Roman Catholic Church, pieces of narwhal tusk, or “unicorn horn,” were often incorporated into bishops’ staffs and architectural designs throughout many medieval churches (Pluskowski, 2004).
**Unicorn Horns**

“Unicorn horn” was also believed to have a healing effect on various ailments. The medicinal properties of “unicorn horn” supposedly cured many ailments, but it was most prized for its ability to detect poisons (Lopez, 1985). It was believed that if one possessed a cup made from “unicorn horn” and a poisoned drink was present in the cup, the cup would perspire, thus alerting its consumer to the poison. With this belief so ingrained in the medieval populations, narwhal tusk was an immensely sought-after commodity by many wealthy patrons and royal dignitaries (McLeish, 2013).

It is believed that the first narwhal tusks were brought to Europe in 1000 CE (McLeish, 2013). Norse traders, who may have hunted narwhals, often brought these tusks to Europe; however, it is believed that the Norse originally obtained the tusks from trading with indigenous groups in the Arctic (Pluskowski, 2004). Even though much of Europe viewed these tusks as evidence of the unicorn’s existence, many of the original traders were unaware of their significance (Lopez, 1985). Narwhal tusk was often sold as whole or as powdered “unicorn horn,” or *alicorn*. With the demand for “unicorn horn” rising, dishonest traders would often sell fake powdered alicorn in the hopes of making a quick profit (McLeish, 2013).

Due to the combined reverence of their faith and their belief in the miraculous powers of “unicorn horn,” many royal dignities and wealthy patrons paid exorbitant sums of money for narwhal tusk. Items incorporating “unicorn horn” were created for the wealthy and included such items as bishops’ croziers and the Duke of Burgundy’s sword hilt (Pluskowski, 2004).
Figure 2 a) Duke of Burgundy's full-length sword. b) Duke of Burgundy's sword hilt made of "unicorn horn." Photos courtesy of Dr. E.C.M. Parsons.

Mary Queen of Scots had a “unicorn” handled croquet mallet, with the tale claiming that she never lost a game while playing with it, and Queen Elizabeth I of England owned a “unicorn horn” valued at £10,000 for the explicit purpose of protecting herself from attempted poisonings (McLeish, 2013). Narwhal tusks were so valuable that often only the royal dignitaries could afford to own one. For example, the Danish royal
throne in Rosenberg Castle was constructed from nine rare and valuable “unicorn horns” (Pluskowski, 2004).

In Scotland, the “biasd na srogaig,” or beast with the lowering horn, was an ungainly sea monster with a single horn in its forehead, which could have been an early Scottish myth of a narwhal (Campbell, 1900). With a present-day distribution of narwhal in close proximity to Scottish waters, an occasional vagrant animal visiting Scottish waters is feasible, especially during the “Little Ice Age” of the latter Middle Ages and
beyond (Parsons, 2004). The unicorn features prominently in the historic Scottish coat of arms, and the occurrence of narwhals, or their tusks, in Scotland might be the reason for this (Bucazaki, 2002; Parsons, 2004).

However, in 1621, a cartographer named Gerhard Mercator, exposed the elaborate merchants’ ruse and concluded that narwhal tusk was ultimately the source of purchased “unicorn horn” (Lopez, 1985). The sale of narwhal tusk, masquerading as “unicorn horn,” was so lucrative that his discovery was immediately dismissed. Roughly 20 years later, Olaus Wörm, a Danish zoologist, corroborated Mercator’s claims in a public lecture. Rather than deter the incessant spread of the unicorn myth, many believed that this further supported unicorn existence for another 100 years (McLeish, 2013).

*Arctic Indigenous Legend*

The Inuit people of Eastern Canada and Greenland, who knew of the existence of the narwhal long before the Europeans, have their own legend of the narwhal. The Inuit in many parts of Eastern Canada and Greenland have continued to orally pass on this legend of the narwhal (McLeish, 2013). Their legend, called “The Blind Boy and the Loon,” tells the story of a son’s revenge against his abusive mother after being healed of his blindness by a kind loon. For his revenge, he ties his mother to his harpoon, and she is dragged to the depth of the sea by a harpooned beluga, where her hair wound around the harpoon line, forming a horn. There the wicked mother is doomed to live the rest of her life as a narwhal (McLeish, 2013).
CHAPTER TWO: DNA EXTRACTION AND PROCESSING METHODOLOGY FOR NARWHAL (*MONODON MONOCEROS*) TUSK

Abstract

The aim of the study was to develop a minimally invasive method of deoxyribonucleic acid (DNA) extraction that is best suited for use on ancient narwhal tusk specimens, especially museum specimens and artifacts of historical significance. This chapter details the methodology used in sample acquisition and treatment, mitochondrial DNA (mtDNA) extraction, the polymerase chain reaction (PCR) primer design, PCR, DNA clean-up, and DNA sequencing. The DNA extraction methodology is a combination of the Rohland and Hofreiter (2007) methods and a modified version of the Qiagen DNeasy Kit (Product Number 69504) (Qiagen DNeasy, 2006). PCR primer design was done through the National Center for Biotechnology Information’s (NCBI) Primer-Basic Local Alignment Search Tool (BLAST), where primers were generated by comparing GenBank’s accession number U72038.1 against the family Monodontidae. Primer pair 4 (NAR-4F and NAR-4R) and primer pair 6 (NAR-6F and NAR-6R) were tested, but primer pair 6 (NAR-6F and NAR-6R) was the only primer pair that amplified DNA during the PCR process due to its smaller base pair (bp) size. PCR products were cleaned up using the USB® product ExoSAP-IT® (Product number 78200) and shipped overnight via FedEx to Eton Biosciences, Inc. (400 Park Offices Dr, Suite 204 Research Triangle Park, NC 27709) for sequencing.
Introduction

Much is unknown about narwhals (*Monodon monoceros*) due to their elusive behavior and their inaccessibility in the heavily iced high Canadian Arctic waters. With a “Least Concern” classification by the International Union for Conservation of Nature and Natural Resources (IUCN) and a total population estimate of roughly 80,000 animals, unlocking the genetic code of this species may be the key to unraveling much of the mystery that surrounds them (Lowry et al. 2017). Since the average lifespan of many marine mammals spans several decades or more, or some even spanning centuries, ancient DNA studies provide an opportunity to study these species (Foote et al. 2012). Mitochondrial DNA (mtDNA) experiences a rate of evolution that is often between five to ten times faster than nuclear DNA, making it the most widely used genetic marker in ancient DNA studies, especially in those pertaining to mammals (Brown et al. 1982; Foote et al. 2012). To date, the mitochondrial cytochrome *b* gene is the most often sequenced for many vertebrates and genetic differences are usually large enough to characterize individual species (Johns & Avise, 1998). For this reason, mtDNA cytochrome *b* was selected for this study.

The tusk of the narwhal is an unusual object, where not only is there a long history of Arctic indigenous communities crafting narwhal tusks into tools and artwork, but also the tusks were very sought after in Europe for over a thousand years (Pluskowski, 2004; McLeish, 2013). As noted in the previous chapter there are many historical artifacts that incorporate ancient and historic narwhal tusks. In addition,
internationally there are many museums that have collected narwhal tusks, initially as curios and latterly as important biological specimens. The genetic makeup of such ancient and historic tusks may help to provide a glimpse into the population genetics of historic narwhal populations. Particularly interesting would be to look at how populations were effected by a changing climate, such as in the mediaeval warm period and the so called “Little Ice Age,” and to look at the effect of climate change on the genotypes of these populations, which could also be used to predict the effects of climate change on current populations (Foote et al. 2012). As noted in the previous chapter, many historic narwhal tusks are incorporated into historical artifacts, and/or are considered to be very valuable (Pluskowski, 2004; McLeish, 2013). The traditional method for extracting DNA from bone is typically quite destructive, e.g. drilling (Rohland & Hofreiter, 2007). Therefore, the aim of the study was to develop a minimally invasive method of DNA extraction that is best suited for use on ancient narwhal tusk specimens, especially museum specimens and artefacts of historical significance.

Methodology

Sample Acquisition and Treatment Methodology

All narwhal tusk specimens were purchased from David Boone at Boone Trading Company (Brinnon, WA 98320). All narwhal tusk specimens were short pieces of unprocessed pre-1972 narwhal tusk (n = 50). All specimen samples were purchased in accordance with the Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora Appendix II and the Marine Mammal Protection Act (MMPA)
of 1972, which states that any narwhal tusk or ivory pieces must be certified as harvested before 1972 (Lowry et al. 2017; Marine Mammal Protection Act, 1972). According to Boone Trading Company (Brinnon, WA 98320), the acquired pieces originated from the J.L. Houston Company before its closure. J.L. Houston Company originally imported narwhal tusks from Pond Inlet, Nunavet, Canada, in the 1960s, where these tusks were then cut into smaller pieces for use in jewelry manufacturing during the Alaskan ivory trade from the 1950s until the 1990s.

Using traditional DNA extraction methods (Rohland & Hofreiter, 2007), bone or tooth samples, such as narwhal tusk, must be ground into a fine powder in order to obtain maximum DNA yield. That is to say, the traditional method is destructive to, or even obliterates, the original bone sample. A control sample using traditional extraction methods was gathered via drilling of the tusk samples with the Proxxon Micromot 50/E drill. The tool’s low gear ratio produces the least heat while in use, which results in less degradation of DNA during the extraction process (Rohland & Hofreiter, 2007). Narwhal tusk powder was removed from the specimens (n = 50) using the Proxxon Micromot 50/E drill and the titanium nitrate coated HSS Twist Drill set with Brad Points of 1.5mm, 2.0mm, 2.5mm, 3.0mm, 3.5mm, 4.0mm (No 28876). Drilling at the lowest speed setting produces a very fine powder and minimal heat. A hole was initially drilled in all of the narwhal tusk specimens (n = 50) and then half of the narwhal tusk specimens (n = 25) were treated with the test method – a minimal grinding of the sides of the sample. This treatment method was believed to have a virtually undetectable effect on the surface of the sample specimen. The DNA was then extracted and compared from the two samples.
**DNA Extraction Methodology**

As noted above, mitochondrial DNA cytochrome *b* extraction was conducted using drilled (control) and ground (treatment) samples from narwhal tusk specimens obtained from Boone Trading Company (n = 50) (Brinnon, WA 98320), using samples as small as 40 milligrams (mg) according to Rohland and Hofreiter (2007). It was determined that the best method for DNA extraction of narwhal tusk is a combination of Rohland and Hofreiter’s (2007) method and a modified version of the Qiagen DNeasy Kit (Product Number 69504) (Qiagen DNeasy, 2006). Initially, each tusk sample was sanitized with 80% isopropanol before drilling or grinding began. After the sample was thoroughly dried from cleaning, in order to minimize the risk of potential injury while drilling or grinding such small pieces of tusk and ultimately to reduce unintended cross-contamination of narwhal tusk samples with human blood, the tusk sample was placed in a clamp to prevent movement.

Following clamping, the Rohland and Hofreiter (2007) method, or the control method, obtained a fine powder from the tusk samples (n = 50) by drilling a hole using a titanium nitrate coated drill bit. In an effort to eliminate any potential cross-contamination between narwhal tusk samples, the Proxxon Micromot 50/E drill, drill bits, clamp, and table were all sanitized with 80% isopropanol after each completion of each control sample and examined to ensure that all residual narwhal tusk powder was removed. The control method typically leaves a very noticeably drilled hole in the specimen and is visually unappealing in samples used for display. The grinding or treatment method obtained a fine powder from the tusk samples (n = 25) by grinding one
side of the sample using a 2.5mm drill bit (No 28876). By applying even pressure during the grinding process, a small, even layer could be stripped off of the sample. Visual examination of the treatment method left the sample looking slightly more polished than before and did not appear to be visually discernable. The sanitized process used during the control method (Rohland & Hofreiter, 2007) was also repeated during the treatment method to prevent any cross-contamination.

Following the Rohland and Hofreiter (2007) method, between 0.04 milligram (mg) and 1.00 gram (g) of the powdered tusk was placed in a sterile 50 milliliter (mL) polypropylene tube, and 20mL of 0.5 molar solution (M) of EDTA, at pH 7.5, was added to decalcify the sample. Powdered tusk weights for each sample can be found in Appendix 1. The tube was agitated on a rocking platform at 37°C for a time period of up to no less than 72 hours and no more than one week, after which the tube was checked to ensure that all of the sample had dissolved. Following the decalcification process, 360 microliters (μL) of Qiagen buffer ATL and 40μL proteinase K were added to each tube. After each tube was mixed by vortexing, the tubes were incubated at 56°C on a rocking platform until the sample was fully lysed; this process could be left for a time period of no less than one day and no more than three days. The samples were then removed from the rocking platform and centrifuged at 2000 x g for 15 minutes, after which the supernatant was discarded.

The Qiagen protocol outlines the remaining steps and those steps are exclusively followed. The Qiagen protocol steps can be referenced on the Qiagen website (https://www.qiagen.com/us/resources/resourcedetail?id=bd4e7285-9329-4158-9207-
Each sample was first vortexed for 15 seconds, before adding 400μL of Buffer AL and vortexing the samples again to ensure thorough mixing. Then 400μL of 100% ethanol was added to the samples and vortexed again to ensure that the solution was thoroughly mixed and homogenous. Following the addition of Buffer AL and 100% ethanol, up to 650μL of the solution, including any precipitate, was pipetted up into a sterile DNeasy Mini spin column that was placed in a 2mL collection tube. Samples were centrifuged at 8000 revolutions per minute (rpm) for one minute, after which the flow-through was discarded and the collection tube reused. Pipetting up to 650μL of the solution was repeated until all of the samples had been loaded into the spin column and spun down.

The previous 2mL collection tube was discarded and the DNeasy Mini spin column was placed in a new 2mL collection tube. Then 500μL of Buffer AW1 was added to the samples, centrifuged at 8000 rpm for one minute, and the flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2mL collection tube, where 500μL Buffer AW2 was added to the samples and centrifuged at 14,000 rpm for three minutes to dry the DNeasy membrane. The flow-through and collection tube were discarded and the DNeasy Mini spin column was placed in a sterile 2mL microcentrifuge tube. Finally, 100μL of Buffer AE was added to the DNeasy membrane, incubated at room temperature for one minute, and the centrifuged at 8000 rpm for one minute to elute. This step was repeated once more for maximum DNA yield and a final volume of 200μL of DNA. Samples were then stored at -20°C until required for further use.
Polymerase Chain Reaction Primer Design Methodology

Early mtDNA studies for cytochrome b in narwhals sought to sample multiple species of cetaceans, including both Odontocete and Mysticete cetacean species, as well as several Artiodactyla species. These early studies focused solely on the evolutionary history of cetaceans as a whole and used two sets of primers encompassing all species for magnifying DNA during the polymerase chain reaction (PCR) process (Milinkovitch, 1995; Arnason & Gullberg, 1996; Arnason et al. 2004). These previously designed primers, shown in the table below, yielded a gene length of 1,140 bp in all tested species (Arnason & Gullberg, 1996). However, these samples were most likely obtained from blood or tissue, which often yields longer bp segments of DNA than bone (Arnason & Gullberg, 1996). Unfortunately, relatively little progress has been made since these studies in understanding narwhal mitochondrial genetics or in designing primers unique to the narwhal.

Table 1 The set of primer pairs that encompassing Odontocete and Mysticete cetacean species, as well as several Artiodactyla species.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Sequence and Source</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light Strand-F</td>
<td>L5’ – AGGCGTCGAGCTTGGACATGAAAAGCCATCGTTG (Arnason &amp; Gullberg, 1996)</td>
<td>1,140</td>
</tr>
<tr>
<td>Light Strand-R</td>
<td>L5’ – ACAGTCGTAAGCTTCAACTACAAGAAGCAYTAATGA (Arnason &amp; Gullberg, 1996)</td>
<td>1,140</td>
</tr>
<tr>
<td>Heavy Strand-F</td>
<td>H5’ – CGGAATTCCATTTTTGGTTTACAAGAC (Arnason &amp; Gullberg, 1996)</td>
<td>1,140</td>
</tr>
<tr>
<td>Heavy Strand-R</td>
<td>H5’ – AAGGAATTCTTTGGGTGGCTGATGGTGGAGT (Arnason &amp; Gullberg, 1996)</td>
<td>1,140</td>
</tr>
</tbody>
</table>
Since DNA extraction was performed on pre-1972, or ancient, narwhal tusk, primers that were shorter in length, rather than longer in length like the previous studies (Milinkovitch, 1995; Arnason & Gullberg, 1996; Arnason et al. 2004), needed to be designed. This is because DNA extracted from ancient samples is often in smaller pieces of roughly 200 bp (Pääbo, 1989). In order to achieve this, mitochondrial gene data for narwhals was obtained for the cytochrome b gene using the National Center for Biotechnology Information’s (NCBI) GenBank. NCBI’s Primer-Basic Local Alignment Search Tool (BLAST) (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome>) was used to generate primers for use in PCR, based on GenBank’s accession number U72038.1 was compared against the genus Monodontidae. Primer pair 4 (NAR-4F and NAR-4R) and primer pair 6 (NAR-6F and NAR-6R) were ultimately chosen as the primers to test for narwhal PCR analysis as shown in the table.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Sequence and Source</th>
<th>Tm (°C)</th>
<th>%G-C</th>
<th>Size (bp)</th>
</tr>
</thead>
</table>
Of the ten initial primers created, primer pair 4 (NAR-4F and NAR-4R) was selected due to its slightly longer 581 bp long segment, whereas primer pair 6 (NAR-6F and NAR-6R) was selected due to its shorter 241 bp long segment. This was done in an effort to determine if bp segments longer than roughly 200 bp could be extracted from ancient samples. Both primer pair 4 (NAR-4F and NAR-4R) and primer pair 6 (NAR-6F and NAR-6R) were ordered from Invitrogen (https://www.thermofisher.com/order/custom-oligo/enterSequences) (Grand Island, NY) on February 7, 2018, for use in PCR. Both primer pairs were tested to determine which one yielded consistent DNA data for ancient genetics in narwhal tusk.

**Polymerase Chain Reaction Methodology**

The Qiagen HotStar Taq Plus Master Mix Kit (Product Number 203643) was used to best amplify the narwhal tusk DNA samples. The master mix, the diethyl pyrocarbonate (DEPC) water, the forward primers, the reverse primers, and the extracted DNA were thawed and vortexed for 20 seconds to ensure even concentration. Sterile 0.2mL strip PCR tubes were labeled and in a 20μL reaction, 8μL of master mix, 7μL of DEPC water, 1μL of forward primer, 1μL of reverse primer, and 3μL of DNA, were added to the sterilized 0.2mL tubes. The 0.2mL strip PCR tubes were centrifuged for one minute after the components were added to ensure thorough mixing of the solution.

The 0.2mL strip PCR tubes were placed in the BioRad T100™ Thermal Cycler (Product number 1861096) which was programmed according to the primer melting temperatures and the protocols outlined by Qiagen. The best program used for all samples of DNA was 96°C for five minutes; 40 cycles of 96°C for 30 seconds, 53°C for one
minute, 72°C for one minute; followed by 72°C for 10 minutes. After testing both primer pair sets, it was determined that primer pair 4 (NAR-4F and NAR-4R) could not amplify ancient DNA from narwhal tusk. Primer pair 4 (NAR-4F and NAR-4R) most likely did not amplify DNA from narwhal tusk due to the fact that ancient samples often only amplify DNA data in smaller pieces of roughly 200 bp (Pääbo, 1989). Therefore, primer pair 6 (NAR-6F and NAR-6R) was exclusively used for ancient DNA amplification of narwhal tusk.
Based on the results of electrophoresis, primer NAR-6 was selected for continued use during this project.

**DNA Clean-up and Sequencing Methodology**

After PCR was completed, a 2% agarose gel was made, where two TopVision Agarose Tablets were added to 60mL of 1X TBE. As the mixture cooled, 2μL of ethidium bromide (EtBr) was added to the mixture for visualization of DNA during electrophoresis. The mixture was allowed to cool to room temperature and added to the electrophoresis gel mold containing eight wells. Once the gel had solidified, 1X TBE liquid was added to the gel mold until the gel was fully covered. Additionally, 5μL of
ladder was added to the first well, a mixture of 3μL of dye and 5μL of each PCR sample was added to the remaining wells. Electrophoresis was carried out and the samples imaged.

This process was done to ensure that bands were present at the expected place on the ladder, confirming amplification of DNA during the PCR process. After electrophoresis imaging confirmed amplification of DNA, the PCR products were cleaned up using the USB® product ExoSAP-IT® (Product number 78200). Sterile 0.2mL strip PCR tubes were labeled and 2μL of ExoSAP-IT® and 5μL of PCR product was added to the sterilized 0.2mL strip PCR tubes; these were then centrifuged for one minute to ensure thorough mixing. The reaction mix was first heated to 37°C for 15 minutes to dephosphorylate the DNA and then heated to 80°C for 15 minutes to inactivate the enzyme.

The DNA was then shipped in 0.2mL strip PCR tubes overnight by FedEx to Eton Biosciences, Inc. (400 Park Offices Dr, Suite 204 Research Triangle Park, NC 27709). This company (https://www.etonbio.com/) was selected for sequencing because this company prefers sequencing DNA using the ExoSAP-IT® clean-up method and produces publication-quality sequencing. Sequences were obtained and then examined using the computer programs SEQUENCHER and MEGA X. SEQUENCHER allows the user to clean up sequences by removing invalid base calls, as well as run multiple alignment of DNA sequences; MEGA X was used to create phylogenetic trees.
CHAPTER THREE: DRILLING VERSUS GRINDING? EXAMINING A MINIMALLY DESTRUCTIVE METHOD OF ANCIENT DNA EXTRACTION TO PRESERVE SPECIMEN APPEARANCE

Abstract

Preserving and maintaining the appearance of collections on display is often the primary goal of most museum curators; however, this stands in stark contrast to the creation of holes usually required during destructive sampling. This chapter compares a drilling method versus a grinding method in order to develop the most minimally destructive method for ancient DNA extraction of narwhal (Monodon monoceros) tusk. Both drilled samples and ground samples amplified mitochondrial DNA (mtDNA) to the same level of accuracy, but the grinding technique ultimately resulted in significant reduction in physical marring of the surface of the samples. Ancient DNA extracted from narwhal tusk appeared to exhibit low levels of underlying background noise in most chromatograms and very little genetic diversity was found in these specimens. Phylogenetic trees confirmed these findings and the specimens formed two outgroups (i.e. separate individuals or populations) containing assorted levels of both drilled or ground samples. Further examination of the chromatograms obtained during this study also revealed the presence of nuclear-mitochondrial DNA (numt) transfers in narwhal DNA. However, future studies in D-loop primer design, microsatellite analyses, and next-generation sequencing technologies, such as pyrosequencing, will be necessary to better
understand the population dynamics of narwhals, as well as identify how many individuals or pods were represented by the narwhal tusks \( n = 50 \) used in this study.

**Introduction**

The study of ancient genetics often conjures up mental images of extracting DNA from fossil fragments, the most famous popular culture reference being the recreation of dinosaurs from ancient DNA from blood preserved in amber in the movie *Jurassic Park* (Spielberg, 1993). While breakthroughs in technology make this a rapidly expanding field, resurrecting extinct species is a far cry from the reality of ancient genetics (Lister, 1994). The study of ancient genetics, and ultimately ancient DNA, has long been plagued by both the quality and the quantity of genetic material present in the sample (Rohland & Hofreiter, 2007). Most ancient DNA is preserved in mineralized tissues, such as bone and teeth, and DNA extracted from these samples is often in smaller pieces of roughly 200 base pairs (pb) (Pääbo, 1989; Campos et al. 2012). Not only does the environmental temperature at the burial site of fossils play a critical role in DNA survival rates over time, but even modern storage conditions after excavation can be detrimental to DNA survival (Pruvost et al. 2007).

In addition to these concerns, while museum curators do permit destructive sampling procedures for ancient DNA purposes, requests for destructive samples have increased as the ability to study genetics has gotten better (Wisely et al. 2004). Destructive sampling of museum quality specimens, while extremely valuable in providing insight into extinct or endangered species, is often in direct contrast to the
museum’s desire to maintain specimens for public viewing (Wisely et al. 2004). However, the current method of ancient DNA extraction from bones or teeth involves drilling holes in the mineralized tissues and ancient DNA extracted from ancient skins involves cutting out a small piece (Lister, 1994; Rohland & Hofreiter, 2007) and both of these methods often impact the physical appearance of museum specimens on display. Narwhal tusks or crafted items from such tusks are frequently curated by museums, and often are valuable historical artifacts. DNA from such artifacts could provide invaluable historical population genetic information about narwhales, in particular how their populations responded to changes is historical climate. However, traditional methods of DNA extraction could severely damage historical narwhal artifacts. This chapter explores an alternative minimally destructive sampling method to extract ancient DNA that best preserves the physical appearance of museum specimens.

Methods

Sample Acquisition and DNA Extraction

As detailed in chapter two, narwhal (Monodon monoceros) tusk specimens were purchased from David Boone at Boone Trading Company (Brinnon, WA 98320) and all specimens were short pieces of unprocessed pre-1972 narwhal tusk (n = 50). Samples testing the traditional drilling method, or the control method (Rohland & Hofreiter, 2007), obtained a fine powder from the tusk samples (n = 50) by drilling a hole using a titanium nitrate coated drill bit. This method typically leaves a very noticeably drilled hole in the specimen. The grinding method, or treatment method, obtained a fine powder
from the tusk samples (n = 25) by grinding one side of the sample using a 2.5mm drill bit (No 28876). Applying even pressure during the grinding process removed a small, even layer from the sample and did not appear to be visually discernable. Chapter two also detailed the DNA extraction process, which was a combination of the Rohland and Hofreiter (2007) method and a modified version of the Qiagen DNeasy Kit (Product Number 69504) (Qiagen DNeasy, 2006).

**Polymerase Chain Reaction and Sequencing Methods**

As detailed in chapter two, after primer design using the National Center for Biotechnology Information’s (NCBI) Primer-Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome), primer pair 6 (NAR-6F and NAR-6R) were used exclusively during the polymerase chain reaction (PCR) of all samples collected in both the control method (n = 50) and the treatment method (n = 25). PCR was carried out with the HotStar Taq Plus Master Mix Kit (Product Number 203643). The master mix, the diethyl pyrocarbonate (DEPC) water, the forward primers, the reverse primers, and the extracted DNA were thawed and vortexed for 20 seconds to ensure even concentration. In 20μL reactions, 8μL of master mix, 7μL of DEPC water, 1μL of forward primer, 1μL of reverse primer, and 3μL of DNA, were added to sterilized 0.2mL tubes. The PCR method followed the protocols outlined by Qiagen and DNA was amplified in the BioRad T100™ Thermal Cycler (Product number 1861096). The program used for all samples of DNA was 96°C for five minutes; 40 cycles of 96°C for 30 seconds, 53°C for one minute, 72°C for one minute; followed by 72°C for 10 minutes.
After PCR was completed, a 2% agarose gel was made, in which electrophoresis was carried out and the samples imaged; this process was done to ensure that bands were present at the expected place on the ladder, confirming amplification of DNA during the PCR process. After electrophoresis imaging confirmed amplification of DNA, the PCR products were cleaned up using the USB® product ExoSAP-IT® (Product number 78200) and following the directions detailed in chapter two. The DNA was then shipped overnight by FedEx to Eton Biosciences, Inc. (400 Park Offices Dr, Suite 204 Research Triangle Park, NC 27709) for sequencing. DNA sequences used in this project were sequenced both forward and backward for increased validity. The bases of each sequence were also examined in the chromatograms for validity; however, it should be noted that almost all narwhal tusk sequences contained some small level of background noise. Only the sequences with high levels of background noise were not used in the analysis.

**Sexing Technique Confirmation**

Two methods of PCR-based sexing techniques, the Konrad et al. (2017) method and the Robertson et al. (2018) method, were tested in this study for confirmation of the specimen’s sex. The primers used in both of these methods targeted smaller sections of the SRY gene and the ZFX gene and the primers used in the Konrad et al. (2017) method were designed for highly degraded cetacean DNA and were therefore the same primers used in this study. However, primers targeting the SRY gene and SFX gene of narwhals were designed specifically for use in the Robertson et al. (2018) methods since the paper focused on pinniped DNA, rather than cetacean DNA. The primers for each method are listed in the table below. All primer pairs were ordered from Invitrogen.
for use in PCR.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Sequence and Source</th>
<th>T_m (°C)</th>
<th>%G-C</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CetSex94-F</td>
<td>5’-AGAGCCACAAGCTGACC-3’ (Konrad et al. 2017)</td>
<td>76</td>
<td>59</td>
<td>94</td>
</tr>
<tr>
<td>CetSex94-R</td>
<td>5’-CATTTTGAGGTAACAAAGCC-3’ (Konrad et al. 2017)</td>
<td>78</td>
<td>36</td>
<td>94</td>
</tr>
<tr>
<td>NARSRY-F</td>
<td>5’-CCCATGAACGCCTTCTATGGTGGA-3’ (Konrad et al. 2017)</td>
<td>93</td>
<td>54</td>
<td>244</td>
</tr>
<tr>
<td>NARSRY-R</td>
<td>5’-GGCTTCCGGACAGGCTGATA-3’</td>
<td>86</td>
<td>60</td>
<td>244</td>
</tr>
<tr>
<td>NARZFY-F</td>
<td>5’-TGGAAGGTTGTCAGGATGG-3’</td>
<td>76</td>
<td>50</td>
<td>168</td>
</tr>
<tr>
<td>NARZFY-R</td>
<td>5’-TGAGAGTGGACACTCAT-3’</td>
<td>80</td>
<td>45</td>
<td>168</td>
</tr>
</tbody>
</table>

The Konrad et al. (2017) method carried out PCR in 20μL reactions in which 1μL of bovine serum albumin (BSA), 8μL of Invitrogen™ Platinum™ SuperFi™ DNA Polymerase master mix (Product number 12358010), 1μL of 25 millimolar (mM) magnesium chloride (MgCl₂), 1μL of CetSex94 forward primer, 1μL of CetSex94 reverse primer, and 8μL of DNA were added to sterilized 0.2mL tubes. The PCR method followed the protocols outlined by Konrad et al. (2017) and DNA was amplified in the BioRad T100™ Thermal Cycler (Product number 1861096). The Konrad et al. (2017) method used the following parameters: initial denaturing at 94°C for five minutes; 35 cycles of 94°C for 30 seconds, annealing at 51°C for one minute, extension at 72°C for one minute; followed by a final elongation step at 72°C for 10 minutes. The restriction digest was performed after amplification of narwhal DNA in 20μL reactions containing 10μL of the PCR product, 8.5μL of Taq 1 buffer (Product number B28), 0.05μL of 0.02%
BSA, and 1 μL of 5 unified atomic mass unit (U) of Taq 1 (Product number ER0671) in sterilized 0.2mL tubes. These tubes were incubated at 65°C for one hour before electrophoresis on a 2% agarose gel stained with EtBr.

The Robertson et al. (2018) method carried out two PCR reactions in 25 μL reactions. The first 25 μL reaction contained the following: 8 μL of HotStar Taq Plus master mix (Product number 203643), 11 μL of DEPC water, 1 μL of 25mM of MgCl₂, 1 μL of NARSRY forward primer, 1 μL of NARSRY reverse primer, and 3 μL of DNA were added to sterilized 0.2mL tubes. The second 25 μL reaction contained the following: 8 μL of HotStar Taq Plus master mix (Product number 203643), 11 μL of DEPC water, 1 μL of 25mM of MgCl₂, 1 μL of NARZFY forward primer, 1 μL of NARZFY reverse primer, and 3 μL of DNA were added to sterilized 0.2mL tubes. The PCR method followed the protocols outlined by the Robertson et al. (2018) methods and DNA was amplified in the BioRad T100™ Thermal Cycler (Product number 1861096). The Robertson et al (2018) method was modified and used the following parameters: initial denaturing at 96°C for five minutes; 40 cycles of 96°C for 30 seconds, annealing at 53°C for one minute, extension at 72°C for one minute; followed by a final elongation step at 72°C for 10 minutes. Both PCR reactions were run through electrophoresis on a 2% agarose gel stained with EtBr.

Data Analysis

All sequences used in this study were labeled “N” for “Narwhal,” the sample number, the method of extraction, with a “DS” for “Drilled Sample” or a “GS” for “Ground Sample,” and the extraction number (e.g. N001DS001 or N002GS002). This
was done to differentiate them from the GenBank published sequences used for reference. SEQUENCHER 5.4.6 (Gene Codes Corporation, Ann Arbor, MI) was used to edit sequences and create alignments of the DNA sequences. All extracted sequences below 70% quality were removed in SEQUENCHER 5.4.6 and only one of the best sequences for both drilled and ground samples were used for analysis.

Since the sequences with the highest data quality appeared to be predominately forward sequences, all reverse sequences were also removed. The following samples with poor sequencing data quality were also removed in SEQUENCHER 5.4.6 and not used for analysis.

<table>
<thead>
<tr>
<th>Samples with Poor Sequencing Data Quality</th>
<th>Drilled Samples</th>
<th>Ground Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>N022DS001</td>
<td></td>
<td>N003GS001</td>
</tr>
<tr>
<td>N032DS001</td>
<td></td>
<td>N005GS001</td>
</tr>
<tr>
<td>N039DS001</td>
<td></td>
<td>N025GS001</td>
</tr>
</tbody>
</table>

The chromatograms for the highest quality data sequences were examined base by base to ensure SEQUENCHER 5.4.6 called the bases accurately. The remaining sequences were aligned into two contigs using the assembly algorithm parameters of a minimum match percentage of 95% and a minimum bp overlap of 70 bp. The resulting contigs were exported as FASTA files for use building phylogenetic trees.

MEGA 7 (Molecular Evolutionary Genetics Analysis) was used to generate the neighbor-joining and the maximum parsimony phylogenetic trees. Sequences from this
study were then compared to published narwhal (*Monodon monoceros*) and beluga (*Delphinapterus leucas*) cytochrome *b* sequences obtained from GenBank. The phylogenetic trees included cow (*Bos taurus*) and hippopotamus (*Hippopotamus amphibius*) cytochrome *b* sequences obtained from GenBank as outgroups. Optimal evolutionary history of the Monodontidae family for the cytochrome *b* gene, as shown below, was inferred using the Neighbor-Joining method (Saitou & Nei, 1987).
Figure 5 The evolutionary relationship of *Monodon monoceros* shown by a neighbor-joining phylogenetic tree using cytochrome b DNA.
The optimal tree with the sum of branch length was equal to 1.52213548. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura et al. 2004). The analysis involved 47 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 120 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Optimal evolutionary history of the Monodontidae family for the cytochrome \( b \) gene, as shown below, was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993).
Figure 6 The evolutionary relationship of *Monodon monoceros* shown by a maximum likelihood phylogenetic tree using cytochrome *b* DNA.
The tree with the highest log likelihood (-599.44) was shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The rate variation model allowed for some sites to be evolutionarily invariable. The analysis involved 47 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 120 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Results

When the primers for the cytochrome b region were originally designed, the primer pair 4 (NAR-4F and NAR-4R) segments were designed to amplify 581 bp and the primer pair 6 (NAR-6F and NAR-6R) segments were designed to amplify 241 bp. Primer pair 4 (NAR-4F and NAR-4R) segments were designed to be longer in an effort to potentially amplify a slightly larger region of the cytochrome b mitochondrial DNA. Unfortunately, primer pair 4 (NAR-4F and NAR-4R) segments were never able to amplify any narwhal tusk DNA during the testing phase. Primer pair 6 (NAR-6F and NAR-6R) segments successfully amplified DNA in all narwhal tusk specimens used during both the drilling (n = 50) and grinding (n = 26) methods to the same level of accuracy. All of the ground samples (n = 26), or 52% of the total sample size (n = 50), amplified and DNA was visible during electrophoresis. This can be seen in the following
image, in which the drilled samples and the ground samples amplified at roughly 250 bp, as expected based on the primer pair 6 (NAR-6F and NAR-6R) design, and as indicated on the ladder.

![Image of DNA gel showing amplified samples at roughly 250 bp](image)

**Figure 7** a) Amplification of the drilled samples at roughly 250 bp. b) Amplification of the ground samples at roughly 250 bp.

Using the Konrad et al. (2017) sexing method for highly degraded cetacean DNA during electrophoresis, females should have two bands of 37 bp and 57 bp and males should have three bands of 37 bp, 57 bp, and 94 bp. Since tusks are predominately found in male narwhals and rarely found in female narwhals (Reeves & Tracey, 1980), the expected results should have been predominately male. Unfortunately, the results from the Konrad et al. (2017) sexing method for highly degraded cetacean DNA were not reproduceable during this study as shown in the figure below.
Using a modified version of the Robertson et al. (2018) sexing method for pinnipeds, the females should not have bands present and the males should have bands present during electrophoresis. Since this protocol required running two PCR reactions, one for the SRY gene and one for the ZFY gene, two gels were run though electrophoresis. Unfortunately, the results from the modified version of the Robertson et
al. (2018) sexing method for pinnipeds were also not reproduceable during this study as shown in the figure below. During electrophoresis of the NARSRY primers in the Robertson et al. (2018) method, the manatee was the only sample visible. This was not an expected result and the manatee should not have exhibited any bands.

![Image](image-url)

**Figure 9** Negative sex confirmation from samples N008DS001 - N012DS001 and a manatee using the Robertson et al. 2018 sexing method for pinnipeds using the SRY gene and the ZFY gene.

Results from both the Konrad et al. (2017) method and the modified Robertson et al. (2018) method were not reproduceable. Both the Konrad et al. (2017) methods and the Robertson et al. (2018) methods failed to provide specific volumetric measurements and amounts used during the PCR reactions were estimated based off of a starting assumption of 20µL reactions. Since no results were visible during electrophoresis in the Konrad et al. (2017) method, the sex of the narwhal was never confirmed. The manatee sample
exhibited multiple bands using the Robertson et al. (2018) method. This was not an expected result so the manatee was excluded from this discussion. No narwhal results were visible during electrophoresis in the Robertson et al. (2018) method; therefore, the sex of the narwhal was never confirmed either. Both of these methods sampled tissue or blood from species and more research would need to be conducted to determine if narwhal tusk samples allow for a sexing technique.

SEQUENCHER 5.4.6 was used to create a multiple alignment for all the cytochrome b sequences matching the two GenBank *M. monoceros* sequences (accession numbers U72038.1 and X92532.1). This alignment resulted in two contigs, each of which contained mixtures of both drilled and ground samples. When ran through NCBI’s BLAST system (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome), 45 drilled samples matched *M. monoceros* at greater than 95%, and 24 of ground samples matched *M. monoceros* at greater than 95%. Very little sequence diversity in the narwhal tusk samples was observed, except in two notable examples, N032DS001_NAR6R_01_31_2019, sequenced on January 31, 2019, and N035DS001_NAR6F_01_31_2019, also sequenced on January 31, 2019.

When run through NCBI’s BLAST database, the sample N032DS001_NAR6R_01_31_2019 matched a nitrogen-fixing soil bacterial *Bradyrhizobium* species found on legumes (Hameed et al. 2004) at 93%. Since sample N032DS001_NAR6R_01_31_2019 was the only high-quality sequence obtained, it was removed from the samples used for phylogenetic trees due to the results from the BLAST search, as well as the fact it was a reverse sequence, while the rest were forward
sequences. When run through the NCBI’s BLAST database, the original, unedited sample N035DS001_NAR6F_01_31_2019 matched as *M. monoceros* at 34%. However, when after cleaning up sample N035DS001_NAR6F_01_31_2019 through editing out the lower quality bases at the beginning and end of the sequence, the sample matched a lactic acid bacterial *Lactococcus* species in the genus *Streptococcus* (Snel et al. 2011) at 63%. The edited sample N035DS001_NAR6F_01_31_2019 was also removed from the sequences used for phylogenetic trees due to the contradicting results obtained from the BLAST search, and replaced with the original, unedited sequence.

Both the neighbor-joining tree and the maximum likelihood tree showed the cows (*Bos taurus*) and hippopotamuses (*Hippopotamus amphibious*) aligned on the outside of the cetacean part of the tree. The Monodontidae family broke off from the cows and hippopotamuses into two species: the belugas (*Delphinapterus leucas*) and narwhals (*Monodon monoceros*). The samples (n = 50) formed two outgroups under the GenBank narwhal references (accession numbers U72038.1 and X92532.1). These outgroups contained a mixture of both ground and drilled samples. The original, unedited sample N035DS001_NAR6F_01_31_2019 formed a third outlying group independent from any of the others.

Despite the underlying background noise present in most sequences’ chromatograms, examination of these chromatograms revealed the presence of nuclear mitochondrial DNA (numt), in which a piece of mitochondrial DNA had been translocated into the nucleus. Numts (pronounced new-mights) were present in at least 30 of the DNA sequences, however it was difficult to determine if numts were present in the
remaining 15 DNA sequences due to higher levels of background noise found in the chromatograms. In sample N001DS002_NAR6F_03_01_2018, at position 31, SEQUENCER 5.4.6 is calling the base as a blue C, but there is also a red T present at the same location, as designated by the black box in the following chromatogram.

![Figure 10](image) Numt location at position 31, as designated by the black box, in sample N001DS002_NAR6F_03_01_2018.

This is also observed in sample N002DS001_NAR6F_03_01_2018, at position 60, where SEQUENCER 5.4.6 is calling the base as a black G, but there is also a green A present at the same location, as designated by the black box in the following chromatogram.

![Figure 11](image) Numt location at position 60, as designated by the black box, in sample N002DS001_NAR6F_03_01_2018.
**Discussion**

This research showed that pre-1972 narwhal (*Monodon monoceros*) tusk amplifies mitochondrial cytochrome *b* DNA in segments roughly 250 bp long. Previous research in early mtDNA studies for cytochrome *b* in narwhals sampled both Odontocete and Mysticete cetacean species and designed primers with segments roughly 1,140 bp long (Arnason & Gullberg, 1996). This demonstrated that previous research focused primarily on extracting DNA from blood or tissue, which often yields longer bp segments of DNA (Arnason & Gullberg, 1996). However, this study suggests that the results obtained during the extraction process are consistent with current research in ancient genetics, which states that DNA extracted from ancient samples is often only amplified in smaller pieces of roughly 200 bp (Pääbo, 1989). By creating several sets of overlapping primers, the entire cytochrome *b* gene could be sequenced.

The research from this study also demonstrated that accurate results are obtained during both the drilling method and the grinding method seen in the figure below.
The drilling method appears more destructive during sampling processes due to the presence of a hole in the specimen, whereas the grinding method, as shown below, does not appear to be as noticeable to the specimens due to grinding the top layer off.
The grinding method produces an effect on the tusk little different from an overenthusiastic polishing procedure. This method could potentially be transferable to other ivory specimens, such as elephant or mammoth tusk. When sampling museum specimens, especially those used for display purposes, it is suggested that the grinding method from this study be implemented during sampling in an effort to prevent visible marring of the sample. Hopefully, this method will accommodate the goals of museum managers to preserve and maintain the samples on display or in the collections (Wisely et al. 2004).
There appears to be very little genetic diversity in the specimens used during this research study. The drilled and ground samples matched *M. monoceros* at greater than 95% when run through NCBI’s BLAST database. However, both the neighbor-joining and the maximum likelihood trees showed two outgroups from the specimens used in this study, suggesting that the samples belong to two different individuals or two different populations of narwhals. Unfortunately, it is difficult to determine this under the parameters of this study, and microsatellite primers for nuclear DNA would need to be designed in order to conduct microsatellite analyses of the data collected. Microsatellite analyses of the collected specimens would determine whether these two outgroups represent two different individuals or two different populations at the species level (Dalebout et al. 2002).

During the matching of sequences in NCBI’s BLAST database, an interesting discovery yielded results in two samples containing bacterial DNA rather than the expected narwhal DNA. The cleaned-up sample N032DS001_NAR6R_01_31_2019 matched a nitrogen-fixing soil bacterial *Bradyrhizobium* species found on legumes (Hameed et al. 2004) at 93% when ran through NCBI’s BLAST database. The unedited sample N035DS001_NAR6F_01_31_2019 matched a *M. monoceros* at 34%, however the cleaned-up sample N035DS001_NAR6F_01_31_2019 matched a lactic acid bacterial *Lactococcus* species in the genus *Streptococcus* (Snel et al. 2011) at 63% when ran through NCBI’s BLAST database. The BLAST results from samples N032DS001_NAR6R_01_31_2019 and N035DS001_NAR6F_01_31_2019 suggests contamination of the specimen, but considering the thorough cleaning process, this seems
unlikely. The lactic acid bacterial *Lactococcus* species in the genus *Streptococcus* are often found in the bacterial cultures from human mouths (Snel et al. 2011). Since narwhal tusk construction is the reverse of human teeth, with the hard enamel on the inside, surrounded by the dentin, and finally the cementum on the outside of the tusk (Nweeia et al. 2008), it is possible that narwhals possess similar oral bacterial cultures. During the drilling process, if a pocket of this bacteria was encountered, it would account for the contradictory BLAST system results, with the sample matching as both *M. monoceros* and the lactic acid bacterial *Lactococcus* species in the genus *Streptococcus* (Snel et al. 2011).

Previous research by Richly and Leister (2004) found that copies of mitochondrial DNA are frequently present in the sequences of several eukaryotic nuclear genomes. Based on the results of that study (Richly & Leister, 2004), it should come as no surprise that the presence of numts was discovered in multiple narwhal tusk specimens. Considering that mitochondrial DNA experiences an evolution rate that is often between five to ten times faster than that of nuclear DNA (Brown et al. 1982; Foote et al. 2012), the presence of numts in the nuclear genome could be used to study the evolutionary history of species, since numts retain ancestral mitochondrial information (Ko et al. 2015). However, in order to better understand the numts present in narwhal tusk sequences, the numt sequence and the mitochondrial sequence will have to be separated using next-generation sequencing technologies such as pyrosequencing (Bintz et al. 2014); something that was not covered in the scope of this study.
CHAPTER FOUR: CONCLUSION

The most interesting line of research in this study successfully demonstrated mitochondrial DNA (mtDNA) extraction of cytochrome \( b \) from narwhal (\textit{Monodon monoceros}) tusk material. Previous research in early mitochondrial DNA (mtDNA) studies of cytochrome \( b \) in narwhals sampled either blood or tissue from both Odontocete and Mysticete cetacean species (Arnason & Gullberg, 1996), but never explored sampling ancient DNA preserved in mineralized tissues, such as that found in narwhal tusk, making this study the first of its kind. The study also successfully showed that pre-1972 narwhal tusk does amplify mitochondrial cytochrome \( b \) DNA in segments of roughly 250 base pairs (bp) long. This is consistent with previous research in ancient genetics which states that DNA extracted from ancient samples is often only amplified in smaller pieces of roughly 200 bp (Pääbo, 1989).

Both the traditional drilling method (Rohland & Hofreiter, 2007) and the grinding method used in this study amplify ancient DNA data to the same level of accuracy. However, the traditional drilling method (Rohland & Hofreiter, 2007) does appear as more destructive on the specimen due to the presence of a hole in the specimen after the sampling process is completed. The alternate grinding method is not as visually noticeable on the specimen since the top layers are ground off and this sampling procedure produces an effect little different from overenthusiastic polishing during
cleaning. The results suggest that the minimally destructive method of grinding should be implemented during destructive sampling of museum specimens in an effort to prevent visible marring of the sample and to better accommodate the goals of museum curators (Wisely et al. 2004).

After examination of each of the chromatograms, low-level background noise appeared in all narwhal DNA sequences. Several attempts failed to resolve this issue and minimize the appearance of this background noise, and it is unclear exactly what causes this noise in narwhal tusk samples. There was very little genetic diversity in the specimens used during this research study and recent research examining the nuclear genome of narwhals supports the results of this study in finding low genetic diversity in narwhal populations (Westbury et al. 2019). However, the decision of the International Union for Conservation of Nature (IUCN) to downgrade narwhals from “Near Threatened” to “Least Concern” on the red list of threatened species ignores the low diversity of this species and the resulting low potential for narwhals to genetically adapt to increasing pressures from a rapidly changing climate (Lowry et al. 2017).

**Future Studies**

One issue with this study is neither the Konrad et al. (2017) sexing method for highly degraded cetacean DNA nor the modified Robertson et al. (2018) sexing method for pinnipeds were reproducible. After receiving multiple negative results from the Konrad et al. (2017) sexing method, primers for the SRY gene and the ZFY gene were specifically designed for narwhals using National Center for Biotechnology Information’s (NCBI) BLAST database; however, multiple trials using the modified
Robertson et al. (2018) sexing method were attempted and produced similar negative results. Both of these sexing methods relied on using DNA extracted from blood or tissue, but confirmation of the sex of a specimen should still be possible using one of these techniques. Since no exact volumetric amounts for polymerase chain reaction (PCR) were published in either study, an assumption of 20µL reactions was made for PCR in both of these studies. These assumptions could have been the reason both studies failed to provide reproduceable results. Future studies should focus on re-examining the primers and methodology used in each of the sexing methods to determine if an error was made at some point, or whether determining the sex of a narwhal specimen is even possible using tusk material. Future genetic studies would also do well to emulate adding the volumetric measurements, as done in this study, into the methodology sections to encourage future reproducibility of the findings.

An interesting discovery during this research showed both the presence of a narwhal DNA and a lactic acid bacterial *Lactococcus* species in the genus *Streptococcus* (Snel et al. 2011) in the DNA from sample N035DS001_NAR6F_01_31_2019 when run through NCBI’s BLAST database. Since genetic material from both species was present, contamination is the logical explanation, but considering the thorough cleaning process, this seems an unlikely explanation. Repeated sampling from the sample N035DS001_NAR6F_01_31_2019 while paying special attention to thoroughly cleaning the specimen should also be considered as a way to completely eliminate possible contamination. However, if the lactic acid bacterial *Lactococcus* species in the genus *Streptococcus* are often found in the bacterial cultures from human mouths (Snel et al.
2011), it is reasonable to suggest that narwhals may possess similar oral bacterial cultures. Future research studies should focus on examining other tusk specimens that show discoloration on the surface to determine if this is an isolated contamination event or if narwhals do indeed possess oral bacterial cultures that can cause cavities.

Previous research studies show the presence of nuclear-mitochondrial DNA (numt) transfers in several eukaryotic nuclear genomes (Richly & Leister, 2004) and examinations of the chromatograms obtained during this study of narwhal tusk confirmed these findings. The presence of numts in the nuclear genome could be used to study the evolutionary history of species, since numts retain ancestral mitochondrial information (Ko et al. 2015). Future research studies in microsatellite analyses and next-generation sequencing technologies, such as pyrosequencing, should be explored to better understand the population dynamics of current narwhal populations as well as historic narwhal populations. Developing more primers for use in creating a full mitochondrial cytochrome b gene would also be useful in determine phylogenetic relationships in narwhal specimens used during this study and would be applicable to future studies using display-quality narwhal tusk specimens from museums. Perhaps even more interesting would be to develop primers for the mitochondrial D-loop, which has a stronger chance to place a sample into a specific narwhal population. Much is still unknown about narwhals but unlocking the genetic code of this species may be the key to unraveling much of the mystery that surrounds them.

However, the main objective of this study was achieved – a minimally destructive process was used that has little effect on the surface narwhal tusk materials. The new
method results in DNA collection that is little different to the traditional destructive extraction method. This method could be used on valuable museum specimens and artifacts, for example the Danish Royal throne or the Duke of Burgundy’s sword hilt, to extract historic DNA from these artifacts that might be used to provide invaluable historical population genetic data for narwhals.
APPENDIX 1

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REFERENCES


Whale and Dophin Conservation Society. (2004). The review of significant trade in the narwhal (Monodon monoceros). *AC20 Inf. 9 Proceedings of the Twentieth Meeting of the Animals Committee* (pp. 1-7). Johannesburg, South Africa: CITES.


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