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VALIDATION OF A MITOCHONDRIAL EXTRACTION AND ISOLATION TECHNIQUE FOR USE IN WILDLIFE FORENSICS

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FORENSIC SCIENCE INSTITUTE

BY

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Abstract

The primary goal of this project is to provide the wildlife forensic science community with a DNA analysis protocol that is consistent across all jurisdictions. To accomplish this goal, three important objectives must be achieved: 1) validation of a mitochondrial DNA (mtDNA) specific extraction, purification, and sequencing method that is robust no matter the species, 2) creation of an internet web interface for the submission and analysis of sequence data from casework samples for data analysis, and 3) creation and curation of a database of mitochondrial reference genomes that are forensically-vouchered. This thesis aims to accomplish the first objective.

Current protocols within the field of forensic wildlife DNA analysis require a complete redesign and validation of protocols every time a new species is encountered during casework. This practice increases the turnaround time of casework dramatically, causes challenges when testifying to findings, and relies on data from non-forensic scientists that may not be at the same high standard required for forensic applications. My research focuses on developing a mitochondrial extraction method that is free of nuclear DNA contamination and absent of primer usage and PCR.

I therefore offer to the wildlife forensic community the first total mtDNA sequencing method that can be used on any animal species and is independent of prior genetic knowledge of the organism in question. The project seeks to aid the wildlife forensic science community by providing a streamlined method for the analysis of DNA which is both quicker and more robust.

Introduction

It is the consensus of most scientists that the Earth is currently in the midst of its' sixth great extinction, and humans are considered to be the main causative factor behind this event (Petrossian et al., 2016). While the leading cause of extinction is the destruction of habitat, in recent years poaching has had a significant effect on several keystone species populations (Clarke & de By, 2013). Each continent on Earth maintains a unique flora and fauna, and many regions of the world are home to species that are found nowhere else. This leads to an unfortunate situation – that what is rare is often valuable. As with most valuable natural resources, many individuals seek to profit from the criminal exploitation of these organisms. In recent years, the prevention and prosecution of Wildlife Crime has become a national and international law enforcement priority. Case law broadly defines "Wildlife Crime" as criminal actions taken against animals and the environment, and can include a variety of actions such as illegal hunting or trafficking of living and dead specimens (Linacre & Tobe, 2011). Today Wildlife Crime is conducted on a global scale (Rosen & Smith, 2010), contributing to the extinction of several species and the endangerment of many human lives (Barron, 2015).

The extinction of a species has numerous negative effects that are far more detrimental than the loss of a population of organisms. Following the loss of a wildlife population, an ecosystem is thrown out of balance. Well-established food webs and partners of symbiotic relationships are placed in jeopardy (Chapin et al., 2000). Humans are affected by changing ecosystems just as much as wildlife. Species extinction has been shown to harm humans in a variety of ways: communities that are dependent on wildlife tourism have suffered monetary loss, shrinking wildlife biodiversity has resulted in an increase in disease outbreaks, and loss of predatory species has been shown to physically change the location of rivers (Keesing et al.,

2010; Sustainable Human, 2014). Furthermore, poaching has been confirmed to be a source of funding for several crime and terrorist organizations. These groups use the profits from wildlife crime to fund military campaigns which displace and murder millions (Kideghesho, 2016a).

Wildlife forensics is a rapidly growing field which serves to fight global wildlife poaching and criminal trafficking activity. The casework encountered in this field is vastly different from the types of cases associated with human forensics. A common wildlife forensics case involves determining the legitimacy of an evidentiary item and the forensic scientist must determine if the item is constructed from biological material. If the evidence is biological in nature, the forensic scientists must determine if the material is from a legal source (Gupta et al., 2011). Typically, a small sample of the evidence is taken from the item in question. This representative sample often is analyzed for the presence of wildlife DNA, and the data is compared to a reference DNA sequence to make an identification. While this may seem straightforward, many issues can emerge during the analysis process. The most significant challenge is the lack of uniformity within the Wildlife Forensics community, and this can limit communication and efficiency (Johnson et al., 2014).

Mitochondrial DNA typing is the most widely used form of testing used for species identification in Wildlife Forensics. That stated, there has been no agreement among federal, state, and local labs regarding which genes on the mitochondrial genome should be used for analysis. As a result, several genes within the mitochondrial genome have been used for species identification in casework, loci within the cytochrome b and cytochrome oxidase (COI) genes being the most commonly used (Linacre & Tobe, 2011). Mitochondrial DNA analysis is a time-consuming endeavor. For each species of interest, a forensic laboratory is required to develop and validate a set of primers for testing. When new species are encountered in casework,

previously developed primers sets are not capable of analyzing these new species, so a full developmental validation is needed. This consumes time and resources that could otherwise be focused on casework. Additionally, the protocols used to amplify and analyze mitochondrial genes have been reported to detect mitochondrial-like sequences contained in the nuclear genome (numts). Analysis of numts generates false data that could misrepresent the value of the evidence in court (Calvignac et al., 2011). Furthermore, when the sample is sequenced and needs to be identified, some labs do not have access to a forensically-vouchered reference samples and are required to use an online database to make an identification. While online databases are an acceptable means for acquiring genomic data, many of these databases have been found to have incorrect sequences and many sequences are not peer reviewed or forensically-vouchered (Ayala et al., 2017).

I therefore propose a solution. The first DNA sequencing protocol for the Wildlife Forensic community that is independent of Polymerase Chain Reaction (PCR). Eliminating the need for PCR removes the dependency on having a primer set for every species that is analyzed. To further eliminate the dependency on PCR, Illumina-based sequencing and *de novo* sequencing assembly will be implemented following the successful outcome of this project. A protocol such as this already has been developed for humans – named Mseek - to study heteroplasmy (Jayaprakash et al., 2015). Along with the absence of PCR and implementation of *de novo* sequencing, this protocol uses exonuclease V to digest linear DNA, leaving supercoiled and circular genomes intact. It is hypothesized that treatment with exonuclease V will preferentially digest the linear nuclear genome and leave the circular mitochondrial genome intact. As it pertains to this project, the removal of nuclear DNA serves several purposes. First, the absence of nuclear DNA removes the chance of numts. Second, Illumina sequencing of the

mitochondrial genome is far more efficient and cost effective when the nuclear genome is absent, as the nuclear genome is far larger and can make sequencing far more laborious. Third, the absence of the nuclear genome allows for rapid bioinformatic analysis of the resulting data.

In this thesis, I will provide examples of wildlife crime and some consequences these crimes have on the environment. I will explain current wildlife laws and the organizations that enforce these laws, and the analytical methods used in wildlife investigations. I will detail the issues seen in DNA databases currently used in wildlife cases and introduce a technique for DNA extraction and purification which I hypothesis to resolve many of the current issues within the field of Wildlife DNA analysis. Finally, I will introduce future projects which are complimentary to the new technique and will resolve other database issues.

Wildlife Crime

Wildlife crime is a lucrative endeavor where the rewards often outweigh the risks of punishment. The chances of being arrested for a wildlife crime are slim, and when criminals have been caught, they have frequently received minimum punishments. Barron (2015) determined that over a five-year period only 4% of those convicted of wildlife crimes in Kenya served any jail time. While Barron's study is only one data point, reports and personal communications with practitioners in the field describe the same scenario worldwide. Add the fact that the monetary value of these animal products is consistently increasing and you have a recipe for a global natural disaster. For example, a single pangolin (a small scaled mammal indigenous to Asia) can sell for $700 U.S. dollars (Challender et al., 2015) and many consumers are willingly to pay thousands of dollars for a single piece of ivory art (Gao & Clark, 2014). The small chance of jail time is little in way of a deterrent when the profit is so exorbitant.

The reasons for committing wildlife crimes are as complicated and vast as the individuals who commit them. One well known motivation is that the high prices of animals and their parts appeal to the poverty-stricken regions many exotic animals inhabit, but often motives are more complex (Duffy et al., 2016). For many cultures, certain items from protected wildlife have significance and prestige associated with them. For example, within the Chinese culture ivory carving is an art form hundreds of years old, and owning ivory trinkets is a symbol of status. As ivory is expensive, dealers advertise its' purchase as a financial investment (Gao & Clark, 2014). Due to the dramatic increase of disposable income of the average Chinese household, a far larger portion of the population can now afford items made from ivory, as opposed to only an elite few (Vandegrift, 2013). To meet the overwhelming demand, several stores carry ivory products, often illegally, and even stores authorized to carry legitimate ivory often deal in black market sources (Yu et al., 2017).

In some areas of South America, locals seek to supplement their incomes by capturing parrots to sell in local markets, often earning only a few USD for each bird (Pires et al., 2016). Parrots have been kept as household pets in the Neotropical region for centuries. However, widespread deforestation as well as increased poaching to meet worldwide demand has led to the threatened extinction of many parrot species in the past few decades (Clarke & de By, 2013). Poaching in the 1980s and 1990s involved smuggling birds into countries such as the United States, who would import thousands of exotic birds every year. Legislation landed a significant blow to the illegal market in the US, but poaching still continues at local levels at unsustainable rates (Pires, 2012). While many governments in regions of South America have outlawed the trafficking of exotic animals, local law enforcement often looks the other way, not considering

this crime to be a serious issue, and often these animals can be sold openly in marketplaces (Pires & Clarke, 2011).

Bile is commonly used in Traditional Chinese Medicine (TCM) and has been for centuries. While ancient Chinese pharmacopeias documented the uses of bile from a variety of species, it was bear bile which was the most prized and remains the most valued and used today (Wang & Carey, 2014). Traditionally, bear bile was collected by hunting, but in recent decades "bear farms" have been developed in Asian countries to meet the growing demand and to take the place of decreasing wild populations. While farms are promoted as alternatives to poaching, wild bears are still hunted (despite laws prohibiting this), as many believe wild bile is superior to farmed (Livingstone & Shepherd, 2016). These farms keep living bears and place tubes in the animals to remove the bile by gravity or suction, causing the bears extreme pain, and resulting in illness or death. While public outrage has led to the closing of some of these farms, many still legally (and illegally) operate, as interest in TCM has led to a worldwide market. Bear bile has become a coveted and valuable product, a single kilogram being worth thousands of dollars (Feng et al., 2009).

The United States has one of the highest rates of illegal wildlife trade (Sonricker Hansen et al., 2012), and is the second largest ivory market in the world (Yu et al., 2017). Buyers in the United States tend to want wildlife paraphernalia for a variety of reasons - one of the main being fashion. However, medicinal and food products also are highly sought (Petrossian et al., 2016). Unfortunately, the United States has not passed much legislature in relation to wildlife trade (Smith et al., 2009), and as long as this major market is highly accessible, it will be more difficult to hinder wildlife crime.

A common issue that has further complicated wildlife trade is the sponsorship of illegal activities by governments, as well as the involvement of organized crime and terrorist regimes. Many countries with high wildlife crime rates often have limited resources, and criminals as well as corrupt officials have taken advantage of this to illegally harvest wildlife (Rosen & Smith, 2010). The East African nation of Tanzania is known as a safe haven for poachers, as many Tanzanian officials profit from the illegal wildlife trade (Kideghesho, 2016b). A major blow to conservation efforts in Tanzania occurred in 2013, when Operation Tokomeza Ujangili (Swahili for "End Poaching") was carried out to counter poaching activity (Kideghesho, 2016a). This operation was quickly shut down due to alleged human rights violations. However, many question the legitimacy of these claims as they began to surface when arrested poachers were implicating government officials (Revealed, 2016). Tanzania's intelligence has come forward naming major officials who are involved in ivory trafficking. These individuals, which include members of parliament, have not been prosecuted as they are protected by the government (EIA, 2014). This is unfortunate, as researchers have been able to trace large amounts of confiscated ivory back to Tanzania (Wasser et al., 2015).

Tanzania is far from the only nation touched by corruption. The border of China and Vietnam is infamous as a hotspot for smuggling ivory (Wyatt et al., 2017), as bribery is a key player in helping criminals circumvent numerous checkpoints around the world (Kideghesho, 2016a). In Indonesia, wild caught reptiles often have been marked as farm raised as a way to bypass hunting quotas, including species which cannot be bred in captivity (Nijman et al., 2012). This is not an unusual occurrence, as often in the wildlife trade legitimate and illegitimate activities are intertwined (Wyatt et al., 2017). The caviar market has been controlled heavily by organized crime, an industry which is leading to a decline in the sturgeon population of the

Caspian and Black Seas. The extent of corruption in the caviar market was made evident when the United States found over half of its' major caviar importers involved in illegal poaching and smuggling activities (Zabyelina, 2014).

It is surmised several terrorist groups are involved in wildlife crime, particularly in African countries. These groups include the Sudanese Janjaweed, The Lord's Resistance Army (LRA), and Al Shabaab (Wyler & Sheikh, 2013). Due to the hostile nature of these groups, it is difficult to know the exact level of terrorist involvement in wildlife crime. Some suspect it to be exaggerated (Duffy et al., 2016), but it is likely that terrorists participate at least somewhat in these crimes due to the aforementioned ease it is to commit them.

The illegal harvesting of wildlife is often done at unsustainable rates and occurs all around the world. The kind of wildlife crime carried out by criminals often reflects the economy of that nation – developing areas tend to provide the wildlife resources, smuggling is performed across international borders, and developed nations buy wildlife goods – though by no means do nations strictly adhere to these roles. Criminals can act with little worry due to poor enforcement of laws and corrupt officials. These actions often have garnered little attention, as they are considered low priority. However, there are consequences in failing to stop these crimes.

The Rapid Acceleration of Species Extinction

Removal of a species (such as by poaching) from an area it has long inhabited can trigger substantial changes on the surrounding biological community and ecosystem. For instance, in areas where there is an overhunting of otters, there has been shown to be an increase of sea urchins (an otter food source). An increased urchin population results in the depletion of the kelp found in the environment (Chapin et al., 2000). Kelp forests are important in the

prevention of erosion, and serve as a home and food source for many marine species, which disappear with the kelp (Steneck et al., 2002). Criminal poaching results in the over harvesting of keystone species and has the potential to not only threaten the species being killed but the entire ecosystem as well.

As mentioned above, parrots often are caught illegally to be sold as pets (Pires & Clarke, 2011). Today, 30% of parrot species face extinction or are threatened (Pain et al., 2006). Research has found parrots to be involved in direct and secondary seed dispersal (Blanco et al., 2015), influencing the growth and evolution of Amazon flora. As the populations of many seed dispersers, including several parrot species, are decreasing due to deforestation and poaching, the tropical ecosystem faces an uncertain future (Baños-Villalba et al., 2017).

Many economies depend on wildlife tourism, which attracts tourists by having an ecosystem unique to the area. Damaging an ecosystem leads to monetary consequences for the humans living in these environments. For example, many of the animals poached in Africa are critical to the livelihoods of the people, as wildlife tourism is a valuable industry which provides employment in many Sub-Saharan nations (Kideghesho, 2016a). A study conducted in 2016 found that tourists were drawn to areas of greater biodiversity, and there was an expectation to see certain animals, such as elephants. Furthermore, the study found predators such as lions and leopards drew bigger crowds (Grünewald et al., 2016). Today both elephants and leopards are in danger of extinction, and the lion is a threatened species (Appendices | CITES, 2017).

The elephant is the largest land mammal, altering the landscape of the areas it roams. Elephants bend trees, dig large holes searching for mineral deposits, create large barren areas of trampled grass, and even create barren paths they will reuse (Haynes, 2012). For years this was construed as destructive, and several thousand elephants have been killed in a controversial

attempt to prevent damage to flora (Owen-Smith et al., 2006). Researchers began to look at the damage the elephant caused and the effects it had on the surrounding landscape. Coverdale et al. (2016) found that the plant life warped by the elephants were unaffected or even positively influenced. For instance, there was an increase of vegetative species diversity underneath canopies damaged by elephants. Smaller herbivore species benefit from the damage as well, as the high canopy vegetation is brought down to lower levels (Kohi et al., 2011). This is not unexpected. Many species have evolved alongside the elephant and its' destructive habits, so it is likely that many have adapted. More research needs to be done comparing areas where elephants dwell and areas where they once lived to get a clearer picture of how elephants influence their surroundings.

When a species disappears, the role it filled in the ecosystem is emptied. While another species may eventually settle into the niche, it is a process which can take millions of years and several other species may suffer in the meantime. This was seen when the moas were hunted to extinction when humans arrived in New Zealand. The disappearance of the moa led to the subsequent dying out of the Haast's Eagle, which hunted the moa for food (Bunce et al., 2005). It can be difficult to document all consequences of a species extinction and there could be many which are unforeseen. It is easy in a world of technology to presume that humans will remain unaffected by the destruction of nature. However, humans rely on much of the natural world – apparent in the fishing industry, where the US alone generated over 5 billion dollars in 2016 (NOAA, n.d.).

Legal Measures to Protect Wildlife

The Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES) is an agreement between various nations concerning the trade of endangered species to

protect natural resources and ensure survival of these organisms (CITES, 1973). Originally, mammals were the primary focus of CITES, but from its' inception plant and other animal species also were included in protective measures (Vincent et al., 2014). Trade was accepted as the immediate threat to these species. There are roughly 170 nations who are members of CITES, meeting every two years to discuss policies and legislature involving trade of wildlife. The members of CITES agree to enforce measures passed, which can include limiting the trade of items such as ivory, or banning the hunting of endangered animals (Abensperg-Traun, 2009).

CITES bylaws group endangered species into appendices depending on how threatened the organism has become (www.cites.org). Appendix I lists severely endangered animals of which commercial trade in wild specimens is prohibited. These animals can be imported and exported in special cases, but export and import permits are required (Smith et al., 2011). Appendix II lists animals who are not facing extinction, but whose trade is monitored to ensure sustainable populations (Abensperg-Traun, 2009). Trade of Appendix II species is permitted, but the exporting country must have a permit (Dickson, 2002). Appendix III is less straightforward. The animals in this appendix are protected by individual countries who are requesting aid from others to ensure species survival (Kiehn & Benitez-Diaz, 2013). To obtain export and import permits for Appendix I & II species, a Non-Detriment Finding (NDF) must be submitted to CITES by the interested party (Parsons et al., 2010). NDFs must show that trading in wild members of a listed species will not jeopardize the species' survival, and must be prepared by a Scientific Authority (SA) (Smith et al., 2011). The SA prepares the report and advises the CITES authorities issuing the permits, referencing biological and ecological factors (Parsons et al., 2010). These appendices are supposed to control trade and protect species. Nations involved in CITES have agreed to protect and regulate trade of the animals listed in these appendices, the

level of protection depending on the appendix (Abensperg-Traun, 2009). In recent years the focus of the wildlife community has turned towards the dwindling elephant population (Dickson, 2002). It is estimated that 20% (100,000) of the African elephant population was poached between 2010 and 2012 (Ripple et al., 2015). One way many have proposed to preserve elephant populations is to ban the sale of ivory. In 2015, the United States and China reached an agreement over banning the ivory trade in their respective countries (The White House, 2015). Several nations have developed and implemented Nation Ivory Action Plans (NIAP), a CITES implementation to combat illegal ivory trade by legislation, public awareness, and enforcement (CITES, n.d.). These legal actions require enforcement, but this is more complicated than it would seem. Some ivory markets are legal, so the simple presence of ivory does not denote a crime. For instance, in Thailand, Asian Elephant ivory is legal to trade and own, but ivory from the African Elephant is not (Ogden & Linacre, 2015).

In response to the endangerment of species closer to home, the United States began to pass several wildlife laws shortly before the creation of CITES, the most notable being the Endangered Species Act of 1973 (U.S. Congress, 1973). The Endangered Species Act recognizes the extinction of several species and the depletion of others. It defines what qualifies a species as endangered, and establishes various measures purposed to protect wildlife (U.S. Congress, 1973). More recently, on July 1, 2013 President Obama issued an executive order to combat wildlife trafficking (Executive Order No. 13,648, 2013). This was followed by a published report concerning wildlife poaching and trafficking (Wyler & Sheikh, 2013), which had been ordered by the Obama administration. However, US involvement on combating wildlife crime is largely dependent on the interests of the administration. Thus, involvement in wildlife issues waxes and wanes as executive power changes hands. For instance, during the current Trump administration

many politicians are working to dismantle parts of the Endangered Species Act, as they argue against its' necessity (Palmer, 2017).

Criticism of wildlife law has not been limited to American politicians. CITES has been heralded for its' conservationist actions (Vincent et al., 2014). It also has been the subject of controversy. Many individuals have claimed that CITES methods are ineffective and do little to combat the issue of habitat loss (A du Plessis, 2000), and tales of failures have been easier to find compared to successes. Some have speculated CITES' failure to stop poaching is due to a misunderstanding of why people poach (Duffy et al., 2016). Often poverty is seen as prime motivation for illegal activity, but as mentioned before, intentions often are more complex. Often nations where high levels of poaching occur have little power and voice compared to other nations, and conservation efforts have been led by well-meaning individuals who have little understanding of the cultures where measures are implemented (MacDonald, 2005). Many nations with a history of being colonized have resentment of conservation laws. This can be traced to colonist invasion of these areas and subsequent banning of hunting for locals. This served a two-fold purpose. Banning hunting and use of natural resources made locals dependent on the colonizing nation for their livelihoods (Duffy et al., 2016). Furthermore, hunting was reserved for wealthy Europeans wishing to game hunt. This often was justified by depicting natives to be heartless killers, while white hunters were viewed as noble sportsmen (Neumann, 2004). Many nations, upon independence, kept conservation laws to the disapproval of citizens (Duffy et al., 2016), and used many of the same military tactics used by colonists to enforce them (Neumann, 2004). This has led to barriers between conservationists and the people. Laws often have been viewed by the populace as an attack on the ordinary citizen trying to get by. For instance, when someone is convicted of wildlife crimes, it is often a poor local hunting for food

rather than wealthier individuals involved in the illegal ivory trade (Kideghesho, 2016b). This has led to resentment and disdain towards conservation efforts.

Wildlife law is complex and has many complications which will not be resolved soon. Understanding the motives of poachers could lead to a more complete grasp of issues that plague conservation. The subject of habitat loss also will need to be addressed, as this is a major threat to biodiversity (Vincent et al., 2014). The United States needs to enforce wildlife law more strictly, a move that can be difficult due to the money needed and the size of the nation. Despite the difficulty, the US has a responsibility as the largest consumer of the illegal wildlife market (Sonricker Hansen et al., 2012). The United States has a large amount of influence on world affairs and strict wildlife protection would send a message.

Wildlife Forensics: DNA Analysis for Species Identification

There are many issues unique to the field of wildlife forensics. Some of these issues include: the inability to interview victims and witnesses, park rangers cannot be everywhere at once, and keeping records on every individual organism is impossible (Linacre & Tobe, 2011). Additionally, there are large numbers of species involved in wildlife crime, unlike the single human species handled in traditional forensics, and convincing governments and individuals of the seriousness of wildlife crimes can be challenging (Pires & Clarke, 2011). Even when laws have been enforced, proving a crime has occurred can still be a struggle. Few laboratories handle wildlife crimes, and when they do, funding for detailed forensic analysis is scarce. Additionally, as difficult as it is to obtain funding for laboratory work, the amount of money needed to protect the wildlife itself is staggering (Dalton & Kotze, 2011). Furthermore, if a crime

is committed and DNA analysis is necessary, there are no standardized methods of DNA testing within the field (Johnson et al., 2014).

For most species identification cases, mitochondrial DNA (mtDNA) is used. The mitochondrion is an organelle located in most eukaryotic cells that contains a genome which is separate and unique from the DNA in the cell's nucleus (Boore, 1999). Mitochondria are hypothesized to have descended from bacterium which was engulfed by the ancestors of modern eukaryotic species. Rather than being digested, the mitochondrion's ancestor began a symbiotic relationship within the host, and as time passed became part of the cell itself (Ettema, 2016). However, the mitochondrion still retained its' own separate, circular genome (Boore, 1999), and contains coding genes for its' own ribosomes (Gray et al., 2001). The organelle is surrounded by a double membrane structure (van der Giezen, 2011) and reproduces by fission (independent of the surrounding cell) (Cherubini & Ginés, 2017) - both traits reminiscent of bacteria.

A cell contains thousands of copies of the mtDNA genome and only two of nuclear DNA (nuDNA) (Friedman & Nunnari, 2014). Not only does a mitochondrion contain many copies of DNA, but it also has a layer of proteins protecting it from the environment (Linacre & Tobe, 2011). As a result of this protection and the numerous copies of the mitochondrial genome, a heavily decomposed sample usually only contains intact mtDNA because the nuclear DNA has been degraded (Kitpipit et al., 2013). This is important to the forensic scientist, as samples containing only mtDNA will often be encountered. Traditional medicine and artwork typically alters animal parts used, and often items are made from biological material which contain mtDNA, but have no or low amounts of nuclear DNA, such as hair or scales (Iyengar, 2014).

While the nuclear genome has several regions which are noncoding, almost all the mitochondrial genome has a known function, keeping the region conserved. The human

mitochondrial genome is roughly 16,000 base pairs and codes for 13 proteins, 22 transfer RNAs, and 2 ribosomal RNAs (Friedman & Nunnari, 2014). The mitochondria of other mammalian species are of similar composition, with only slight variation (Kolesnikov & Gerasimov, 2012). For example, although the cat and water buffalo are different species, their mitochondrial genomes are remarkably similar (Kolesnikov & Gerasimov, 2012; Michelizzi et al., 2010; Figures 1, 2). This similarity is due to the fact that the mitochondrial genome is passed to the offspring through the maternal line and there is no recombination (Van Der Bliek, 2016). However, mutations have and continue to occur, leading to mitochondrial genomes that are species specific. Furthermore, as a species disperses throughout an area and reproduces, further alterations occur due to genetic isolation. This phenomenon has provided scientists a way to track migration patterns. For example, Bertola et al. (2015) used mtDNA to trace the evolutionary heritage of lions. By sequencing populations throughout Africa and India, researchers were able to group the lions into four subspecies, and further could determine the country of origin. Autosomal microsatellites were later sequenced and the same bloodlines were determined, supporting the mitochondrial evidence (Bertola et al., 2015). This ability to establish origin is beneficial to forensic investigators. “Poaching hotspots” have been pinpointed using nuclear microsatellites in DNA taken from confiscated elephant tusks (Wasser et al., 2015). It is possible that mtDNA can be implemented in this respect as well.

The mitochondrial genome has a handful of regions which are commonly used for identification: one of the most common, especially in mammals, being the cytochrome b gene. This gene is species specific and has been used to construct phylogenetic trees (Johnson et al., 2014). It was used by Singh et al. (2012) to identify the origin of venom crystals by comparing sequences obtained from the crystals to reference samples in a database. The DNA was extracted

from the crystal and, using universal primers, the cytochrome b sequence was isolated. Comparing the cytochrome b sequence with sequences in the National Center for Biotechnology Information (NCBI) database, it was confirmed the crystals had originated from the Indian Cobra, a protected species (Singh et al., 2012). Another marker, COI, has become increasingly common in recent years and is the focus gene for the "barcode of life" project (Hebert et al., 2003). Other mitochondrial markers used include the RNA genes, and for research of individuals within a species, the D-Loop or control region (Johnson et al., 2014)

Mitochondrial DNA extraction and amplification has unique challenges, notably the phenomenon of nuclear mitochondrial DNA sequences (or numts). Numts are, as the name suggests, mitochondrial sequences that have been copied into the nuclear genome (Gaziev & Shaikhaev, 2010). While there is still some uncertainty of how Numts are formed, it is commonly hypothesized mitochondrial sequences are integrated into the nuclear genome during double strand break repair (Buhay, 2009). Numts are problematic as they tend to be collected during PCR and Sanger Sequencing and, as a result, errors occur in analysis. In some instances, the forward primer will amplify a different sequence than the reverse primer, which can generate unusable data (Buhay, 2009). Numts have led to many erroneous sequences being entered into databases, and research has been affected by this misleading information. Extreme cases can result in false evidence for nonexistent species (Calvignac et al., 2011). Sequences generated from PCR amplified fragments must be carefully proofread. This can be done by ensuring all mitochondrial genes are intact (as numts tend to acquire mutations the original conserved sequence does not), checking peaks on the resulting chromatogram, or inspecting the size of the sequence as numts tend to be shorter than actual mitochondrial sequences (Triant & Hayes, 2011). Performing these checks can be time consuming and are not guaranteed to identify numts.

For instance, one human numt is measured at 14,654 bp (Mourier et al, 2001), nearly the size of the human mitochondrial genome, thus rendering size inspection less useful.

Mitochondrial DNA Analysis using Mseek

As numts can be amplified during PCR, it is ideal to develop a method of extraction and sequencing that excludes amplification by this method. To study heteroplasmy in humans without the contamination of numts, Jayaprakash et al. (2015) developed a protocol with this in mind, which they have named "Mseek". As Mseek was designed for optimal mtDNA output and minimal nuDNA, numts have little chance of being integrated into the mitochondrial sequence. Additionally, the Mseek protocol followed by Illumina sequencing is independent of PCR, thereby removing the need for primers and their respective bias. The Mseek method not only removes numts, but also greatly reduces the size of the sample being sequenced because the nuclear genome has been degraded. The mammalian mitochondrial genome is much smaller than any eukaryotic nuclear genome (Corradi et al., 2010). Removing the nuclear DNA from samples would leave more space on the sequencing platform for the mitochondrial samples to be sequenced in one run, saving time and money.

Next Generation Sequencing

Sequencing in wildlife forensics has traditionally been done by Sanger Sequencing, which has been a reliable but slow and somewhat expensive process. The Sanger method also has limited sensitivity, and *de novo* sequencing is impossible as a complementary oligonucleotide is necessary to begin the process (Behjati & Tarpey, 2013). However, in recent

years, new forms of sequencing have been developed, referred to as "next generation sequencing." While next generation sequencing has steadily replaced Sanger in many research laboratories, the potential in forensics has largely gone untapped.

The Nextera kit will be used to prepare samples libraries, and the NextSeq 500 system will be used to sequence the samples. The NextSeq 500 system uses the Illumina sequencing technique "Sequencing by Synthesis" (SBS). SBS is a parallel sequencing method, meaning several identical strands are sequenced at once. The preparation kit prepares the sample by fragmenting the DNA strand into several smaller sections and adding adapters to the ends. Next, indices (small strands used for referencing), binding sites, and oligos are added.

Cluster amplification takes place on a specially designed slide (see Figure 3). This slide has eight lanes which contain two types of oligos. As the fragments encounter the slide, the adapters at the end of the fragments attach to the oligos and polymerases create a complement strand. The original fragment is washed away. The free end of the remaining strand attaches to the other oligo, forming a bridge. Polymerases form a complementary strand, and the fragments are denatured at one end, leaving two strands to be used for more amplification. After amplification, reverse strands are severed. The 3' end of the remaining forward strand is obstructed to keep priming from occurring. Sequencing involves the use of fluorescent tags unique to each nucleotide. Sequences are determined by the unique emission of each nucleotide and recorded (Illumina, 2010). Fastq files containing the full sequence of the mitochondrial genome will be obtained and used to assemble the genome de novo.

DNA Databases

Many DNA databases have been developed as tools to help researchers, one of the most utilized being the GenBank database administrated by the NCBI (Medicine, 2004). The GenBank database contains nuclear and mitochondrial DNA, and RNA sequences of various species. However, there are many issues with this database. While many of the database entries are from peer reviewed articles, there is no guarantee the sequences are correct, and many are submitted with no form of review (Ayala et al., 2017). Despite this, once a sequence is entered it is assumed it is correct and can be used in primer creation and BLAST searches. Studies have found GenBank to be rife with human contamination (Longo et al., 2011), and in some cases there is as much as a 90% error rate (Tripp et al., 2011). Many species also are largely ignored, containing few or no sequences, and many have yet to have the entire mitochondrial genome sequenced. When a species is absent from the database, researchers investigating a crime must obtain a confirmed specimen and generate a reference sequence, which can take considerable time if the species is protected by law.

Another issue that has plagued many databases is the absence of a known sample vouched for by a qualified forensic scientist. For a specimen to be appropriately vouchered, it must be identified by an expert in the respective field, and should be considered typical for the species (Culley, 2013). This is not a requirement for entry into the NCBI database, and museums are reluctant to allow specimen to be handled. On another note, some museum collection pieces have been found to have been misidentified. Vouchered specimens are routinely sequenced by individual labs, however, access to these specimens has been difficult to obtain (Ogden & Linacre, 2015). It is ideal to have a stored vouchered collection which is accessible to researchers

and investigators. A vouchered specimen serves as a basis of identification for other members of the species and collected vouchers can be used as reference and in research (Culley, 2013).

ROWIN

To assist with the analysis and storage of genomic data, the Forensic Science Institute at the University of Central Oklahoma is in the process of developing the Rapid Online Wildlife Information Network (ROWIN). ROWIN is a bioinformatics pipeline which will provide researchers and investigators with easy access to desired genetic information. To ensure reliability and give freedom to the user, the individual using ROWIN would have complete control from the beginning of the analysis to the end.

The series of steps that will need to be followed to obtain reliable results are displayed in Figure 4, which shows the Mseek protocol. Data from Illumina sequencing will be entered into the database, where the program Velvet (Zerbino, 2010) will begin to assemble the genome. Annotation will be performed by MITOS (Bernt et al., 2013). This will allow the user to freely choose the region of interest within the genome. The marker, going through the program Phylogeny.fr (Dereeper et al., 2008), will be compared to the sequences of forensically vouchered specimen, a phylogenetic tree will be constructed reflecting the identification of the genome in question, and statistical analysis of the sequence will be provided. This provides data which can be used for the courtroom, or for research. Validating the Mseek protocol for use in forensic cases will be the first goal in building the database and ROWIN pipeline.

Materials and Methods

This research aims to validate the Mseek method for use in wildlife forensics. As the protocol originally was designed for humans, there is a need to test its effectiveness on other species, starting with other mammals. DNA extraction was performed via the Epicentre Protocol (MC85200). The nuclear genome will be removed using Exonuclease V. New England Biolab's (NEB) Exonuclease V is a nuclease designed to digest linear DNA by uncoiling and digesting along both the 3' and 5' ends. It was obtained from *Escherichia coli*, being derived from the RecBCD enzyme, which acts as both a helicase and a nuclease. The RecBCD enzyme is involved with recombination and repair (Chang & Julin, 2001). Modified for eukaryotic research, Exonuclease V keeps super coiled and circular DNA intact, making it preferable for mitochondria isolation (New England Biolabs, n.d.). Due to the large ratio of nuclear to mitochondrial DNA, two separate digestions must be performed. I aim to gauge the effectiveness of the enzyme on varying amounts of DNA. Purification will be performed after each digestion using the AMPure system (Beckman Coulter, 2013). For quality control purposes, the samples will be amplified by PCR and run on a gel to observe progress; however, this will not be necessary when used in fieldwork and these amplified samples will not be sequenced. After samples have underwent nuclear digestion and purification, they will be sent off for sequencing.

Total DNA Extraction from Animal Liver

Samples from *Geomys bursarius* and *Geomys breviceps* (pocket gopher) were donated by University of Central Oklahoma's Dr. Michelle Haynie and her team for this project. Dr. Michelle Haynie obtained IACUC approval under application #14011 ("Cryptic Species and Species Boundaries: Using Molecular Markers to Assess Genetic Boundaries in the

Morphologically Cryptic Genus Geomys (Pocket Gophers))". An IACUC exemption application (#16014) was filed for this project and granted. G. *bursarius* and G. *breviceps* were ideal organisms for my pilot study as they are not endangered and do not need protection by CITES. Liver tissue samples were chosen due to the high concentration of mitochondria typically found in the organ (Degli Esposti et al., 2012). A homogenization buffer was made following the Homogenization Buffer A protocol developed by Cold Spring Harbor Laboratory (Clayton & Shadel, 2014). Using a Potter-Elvehjem tissue grinder and the homogenization buffer, 1-5 mg of liver tissue was ground into a liquid, making it suitable for extraction. The homogenizer was kept in an ice bath to prevent premature cell lysis.

For mtDNA extraction, 500 µl of the homogenized tissue was processed using the Epicentre Protocol (MC85200). As per the protocol, the sample was transferred to a centrifuge tube, and 1 µl proteinase K and 300ul of Cell and Tissue Lysis Solution were combined and then added to the sample. The solution was mixed thoroughly. The sample was incubated at 65° C for 15 minutes, and vortexed every 5 minutes during the incubation. The sample was cooled to 37ºC, and 1µl of 5µg/µl RNAse A was added and mixed thoroughly. It was incubated at 37ºC for 30 minutes and placed on ice for 3-5 minutes. The sample was removed, and 175 µl of MPC Protein Precipitation Reagent was added to 300 µl of lysed sample and vortexed vigorously for 10 seconds. The sample underwent centrifugation at 4°C for 10 minutes at ≥10,000 x g in a microcentrifuge. The supernatant was transferred to a clean microcentrifuge tube and the original tube discarded. To the recovered supernatant, 500 µl of isopropanol was added. The tube was inverted several (30-40) times. The DNA was centrifuged at 4°C for 10 minutes in a microcentrifuge. The isopropanol was poured off, ensuring the DNA pellet remained intact. The sample was rinsed twice with 70% ethanol, and centrifuged if the pellet became displaced. The

residual ethanol was removed with a pipette. The extracted sample was resuspended in 100 µl of TE Buffer and checked for quality via spectrophotometry (Epicentre, 2012). The sample was heated to 70ºC for 30 minutes to suspend any Proteinase K activity. Following the suggestion of Jayaprakash et al. (2015), the treated sample underwent PCR and was tested by gel electrophoresis to ensure the presence of both mtDNA and nuDNA at this stage.

Nuclear and Mitochondrial PCR Amplification

The samples underwent PCR with both nuclear and mitochondrial primers at the end of every step to demonstrate mitochondrial isolation. One microliter of DNA was combined with 1.2 µl each of reverse and forward primer, 0.2 µl of Taq, 3.5 µl of buffer, and 17.9 µl of water. The buffer was Buffer F acquired from the Epicentre Failsafe PCR premix kit.

The primers designed for the mitochondrial genome of the species *G. breviceps* were forward 5′-CCC GAG GTA ACA CAA GGC AT-3’ and reverse 5’-AAA AAC CTT GTG AGG GGT GG-3’ and were designed using sequences published by Welborn and Light (2014). For *G. bursarius* mitochondrial primers were forward 5’-CTT CTC CGT GGA CAA AGC CA-3’ and reverse 5’-GTT GAG TGG GTT TGC GGG TA-3’ and designed using sequences published by Genoways & Hamilton (2008). For the nuclear amplification, the universal forward HIST2H4 5’-TSC GIG AYA ACA TYC AGG GIA TCA-3’ and reverse HIST2H4 5’-GTI ACI GTC TTS CKY TTG GCG TGC TC-3’ primers were used (Pineau et al., 2005). The thermocycler was set to 94^0C for 2 min, then 40 cycles of 95^0C 15 sec, 54^0C 1 min, 72^0C 1 min 10 sec. After the 40 cycles, it held for 10 min at 72^0C, and then at 15^0C until removed from the machine.

Exonuclease V Treatment for mtDNA Enrichment

Three different amounts of DNA were evaluated in triplicate to determine the extraction thresholds – 4000 ng, 6000 ng, and 8000 ng. For each treatment, 4 µl of New England Biolab's Exonuclease V (M0345S) was added to 4-8 µg (in 35 µl) of extracted DNA sample, along with 6 µl of New England Biolab's 4 10x buffer, 12 µl 10 mM ATP, and 3 µl of water. The digestion was performed for 48 hours at 37ºC, then the enzyme was inactivated by raising the temperature to 70ºC for 30 min. After digestion, a subsample was collected, and PCR was performed using the nuclear and mitochondrial primers listed above. The sample was purified using Agencourt® AMPure® XP beads. The bottle was shaken to resuspend magnetic particles. AMPure beads and the sample were mixed together in a centrifuge tube based on the guidelines within the manufacturer's protocol (Beckman Coulter, 2013), and pipette mixed together 10 times. The mixture then sat at room temperature for 5 minutes before being placed on a magnetic stand and left to separate for 2 minutes. After the solution cleared, the clear supernatant was pipetted out while the tube remained on the stand. Care was taken to not disturb the beads. With the tube still on the stand, 200µl of 70% ethanol was added and incubated at room temperature for 30 seconds. The alcohol was removed and discarded. Additional ethanol was added and discarded for a second time. The sample tube was removed from the magnetic stand and 40µl of elution buffer was added. The sample tube was placed back on the magnetic stand for one minute, and the elute DNA was transferred to a new tube. Following this first purification step, a subsample was collected, and PCR was performed using the nuclear and mitochondrial primers listed above.

Each sample was treated to a second digestion step that lasted for 16 hours at 37ºC and was inactivated using the same protocol as the first digestion. The purification after the second digestion was carried out in the same manner as the first purification. Following both the second

incubation and purification steps, a subsample was collected, and PCR was performed using the nuclear and mitochondrial primers listed above. In total, four nuclear and mitochondrial PCR reactions were performed on each of the samples.

Results and Discussion

To confirm that the samples had viable mitochondrial and nuclear DNA, extraction was performed, and PCR was run using mt and nu primers. The results of the post-extraction amplification run are displayed in Figure 5. Both nuclear and mitochondrial DNA were present, yet the nuclear DNA fragments were slightly smaller and less intense (contained fewer amplicons. The Epicentre system was designed for optimal mtDNA extraction, so it was expected that the mtDNA would remain more intact than the nuclear counterpart. Nuclear DNA was still present in copious amounts, and the mitochondrial genome and large fragments of nuclear DNA would have both been present for PCR or *de novo* sequencing had this technique stopped at extraction. PCR would have possibly picked up numts and the issues associated with them. If de novo sequencing had been performed at this stage, the nuclear sequences would have been sequenced as well, generating enormous amounts of data which would have been of little interest to the study. The nuclear DNA would also have taken up space which could have been used for the mitochondrial genomes of other specimens.

The samples from the extraction were diluted into three DNA amounts- 4000 ng, 6000 ng, and 8000 ng to test the effectiveness of Exonuclease V on different starting quantities. The samples were treated with the enzyme and PCR was performed once again with the mitochondrial and nuclear primers (see Figure 6). This was a quality test performed to ensure the enzyme was not digesting mitochondrial DNA, and to monitor the effectiveness of the following

purification. The nuclear DNA was still present, albeit in smaller fragments. While the nuclear DNA was beginning to degrade, there was still too much remaining which would potentially interfere with sequencing.

Purification and PCR were performed, and products were run on an agarose gel (Figure 7) to ensure that purification was removing only undesired components. Purification involved the use of Agencourt® AMPure® XP beads. These beads bind to DNA fragments and a magnetic stand is used to separate the beads (and attached DNA) from unwanted components. The undesired articles were pipetted out and the DNA was detached from the beads via a buffer (Beckman Coulter, 2013). The mtDNA samples were present in varying degrees – the 8000 ng sample was the most visible, the 4000 ng the least. The nuclear amplified samples were less visible than any mitochondrial amplified sample, but still present. While there was a small difference in brightness among the nuclear amplified samples, the differences were not as pronounced as with the mitochondrial amplified samples. There was less nuclear DNA than mitochondrial DNA.

The second enzyme treatment was carried out, followed by the second purification. These were done according to the same protocols as the first digestion and purification. As there was evidence Exonuclease V and the purification were performing as expected, quality assurance was only run after both the second enzyme treatment and purification. Figure 8 shows no visible nuclear DNA- the mitochondrial amplified fragments are visible. This indicates the procedure was successful in degrading nuclear DNA while mtDNA remains intact. This result has far reaching implications. As nuclear DNA had been digested and was in minute amounts, the chance of numts infiltrating sequencing was lowered significantly. As numts have been

problematic by generating false mitochondrial genome sequences, minimal nuclear DNA assures more accurate data.

PCR was only performed in this research as a quality control measure to demonstrate the protocol was working as expected (Figures 5-8). To remove primer bias, PCR would not be performed on samples to be sequenced. PCR amplification is unnecessary as bridge amplification is used in next generation sequencing. This can be done without any prior knowledge of the sequence, ensuring more objective data. PCR should only be used for quality control, and no PCR treated samples should be used for next generation sequencing.

The goal of this project was to evaluate the Mseek protocol for application to Wildlife Forensic DNA analysis. This Mseek technique had previously been tested and shown to work for human mitochondria - the aim here was to apply it to other mammals. In short, my pilot study was successful, but a full developmental validation study is needed before the methods described within the study would satisfy the *Quality Assurance Standards for Forensic DNA Testing* criteria. While this was a pilot study and additional sampling is need, I am confident that the Mseek protocol followed by Illumina sequencing will provide ample data for species identification. As demonstrated, gel electrophoresis revealed the gradual degradation of nuclear DNA after each Exonuclease V treatment and purification step. The data shows the dramatic reduction in the nuclear genome, decreasing the likelihood of numts, a problem which has greatly affected the quality of research and forensic investigations in the past. Additionally, the preferential digestion data demonstrates that next-generation sequencing methods are a plausible form of analysis for Wildlife Forensic DNA laboratories. The reduction of the nuclear genome allows several mitochondrial genomes to be sequenced at once, saving time and money. Because

obtaining funding for forensics is an ongoing issue, this can be beneficial for federal, state, and local laboratories.

Conclusion

Having shown that the technique can work on non-endangered mammals, the Mseek method now needs to be tested on yet more species and at diverse levels of detection. More mammalian and non-mammalian species should be tested - particularly endangered species. Because samples used in Wildlife Forensic cases often will be degraded (i.e., powders, carvings), the lower threshold needs to be determined for the Mseek method. The effectiveness on tissues such as ivory and bone should be tested as well, along with other tissues that may be encountered in wildlife investigations. As mammals are not the sole group to endure criminal activity, the Mseek protocol should be tested on other taxa, especially amphibians, whose populations have seen severe declines in past decades (Bower et al., 2017).

The control region of the mitochondria is the most prone to mutation. As a result, this is the area often sequenced when studying population genetics (Walther et al., 2011). Not only does the protocol have potential to be used in research concerning individuals, the planned database could serve as a resource for forensic vouchered control regions. This would be one component of the ideal situation: a forensically vouchered database of DNA sequences which would be accessible to any who wishes to use it.

Though much of the focus of wildlife forensics is on fauna, illegal deforestation is a worldwide issue (Dormontt et al., 2015). As the protocol is designed to work on mitochondria, it is possible that it also could be implemented on chloroplasts. Chloroplasts are theorized to have evolved similar to mitochondria, and have a similar circular genome (Hirano et al., 2016), though there are some key differences. Both organelles are in plant cells, giving researchers more potential genetic material. If validated, the protocol could be used on cases involving timber, or research concerning plant life.

While all mitochondria are hypothesized to have evolved from the same ancestor, impressive variety exists among descendants. Bivalve species, for instance, have separate male and female mitochondrial genomes (Kolesnikov & Gerasimov, 2012), and some species have genomes which undergo recombination (Galtier et al., 2009). The mitochondrial genomes of these species should be extracted and sequenced using Mseek to observe any unique situations that may arise. Linear mitochondrial genomes exist in some cnidarian species (Boore, 1999), and other species have mere remnants of mitochondria in the forms of organelles called mitosomes and hydrogenosomes (van der Giezen, 2011). There is currently little information on these organelles, so there is uncertainty of the impact, if any, they would have on forensic investigations. In one extreme case, one microbial eukaryote (*Monocercomonoides* sp. PA 203) was discovered to have lost its' mitochondria via evolution (Karnkowska et al., 2016). Some of these species are protected by CITES and have the potential to be involved in forensic cases. It should be noted that Mseek will not work on organisms lacking circular genomes, whether located in the nucleus or other organelles. However, the diversity in organisms containing circular mitochondrial genomes could lead to engaging research regarding this method.

Presented in this thesis is an extraction protocol which is time saving, cost effective, and free of nuclear sequences. Biases related to PCR and primers are removed from the procedure, allowing for objectivity, a crucial part of any forensic case. Besides forensic use, the protocol would provide a more objective approach for research use. However, to be used to full potential, there is much work to be done regarding this protocol. It is recommended that numerous samples be tested for more complete validation. Other mammalian species should be tested with this method and eventually other organisms should be tested as well. However, this is the beginning to the construction of a wildlife database and pipeline which will give freedom to the user to

choose their reference sequence and receive crucial bioinformatic information. With diligence and determination, a universal technique and database can be brought to the world of wildlife forensics.

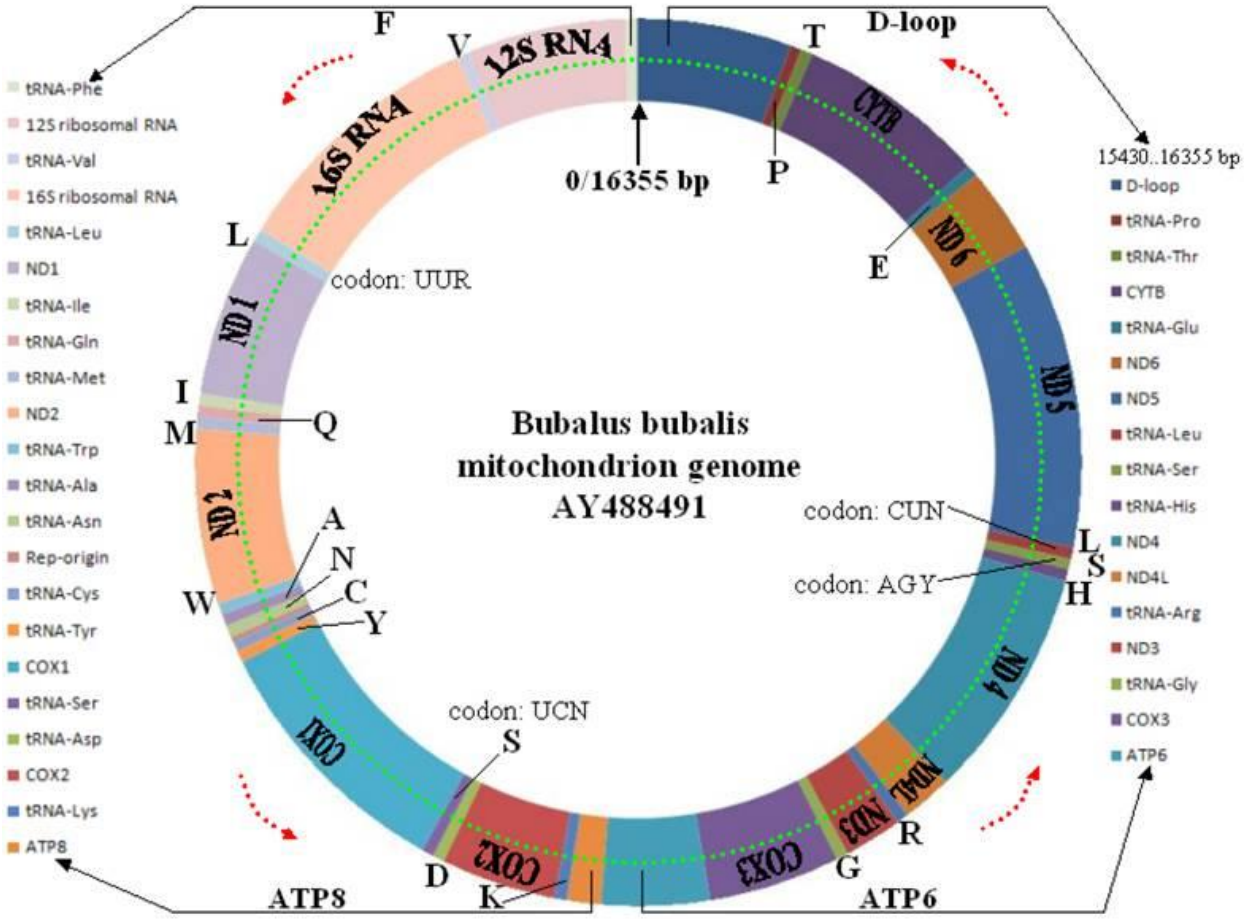


Figure 1: Mitochondrial Genome of *Bubalus bubalis* (water buffalo)
The water buffalo mitochondrial genome is 16355 bp long and contains 37 genes. These include two ribosomal coding genes, 22 for coding tRNA, and 13 for proteins. (Michelizzi et al., 2010)

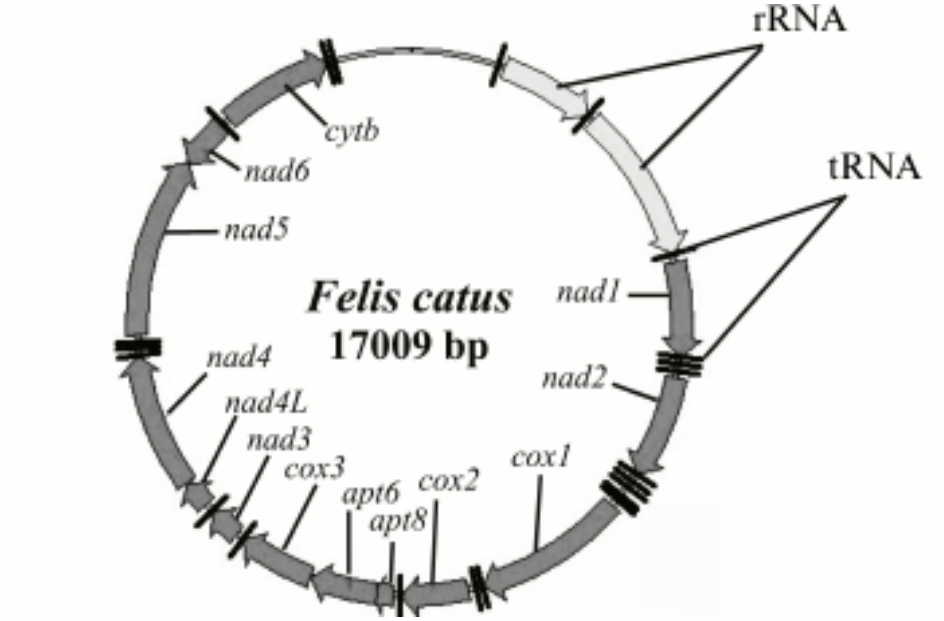


Figure 2: Mitochondrial Genome of *Felis catus* (domestic cat) (Kolesnikov & Gerasimov, 2012) The mitochondrial genome of the domestic feline is 17009 bp long and contains 37 genes. Similar to the water buffalo (and other mammals), the genome contains 2 rRNA genes, 22 tRNA genes, and 13 protein coding genes (Lopez et al., 1996).

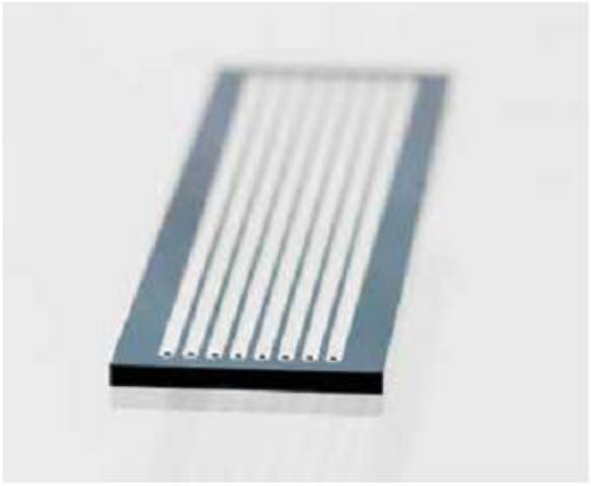


Figure 3: Illumina Flowcell
Next generation sequencing uses a specially designed slide containing lanes. Within these lanes are primers and adapters used in cluster amplification (Illumina, 2010).

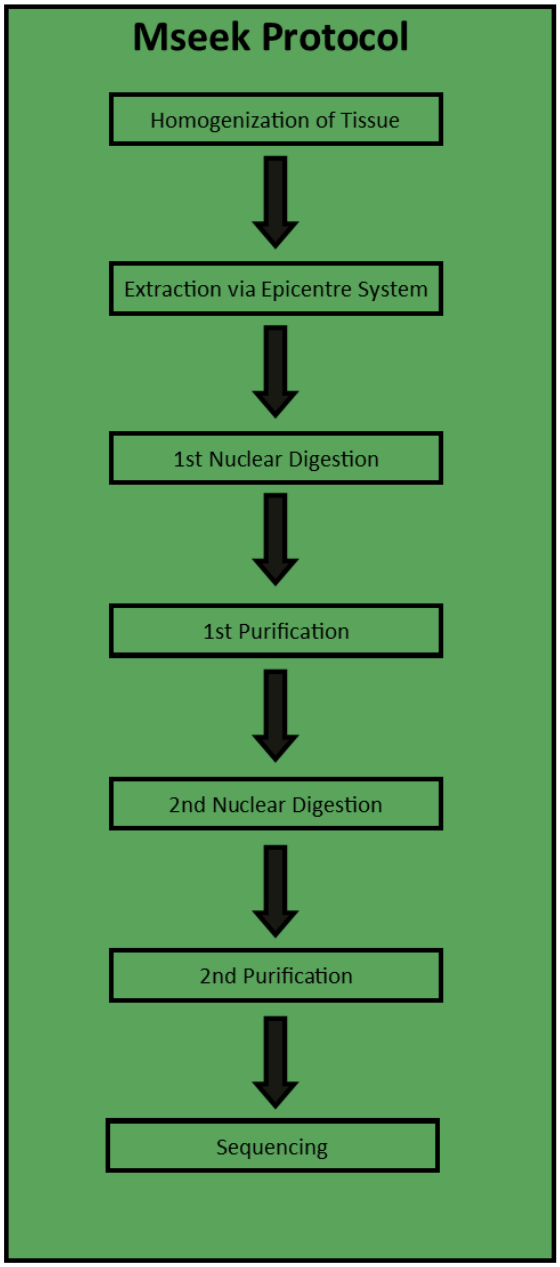


Figure 4: Flowchart of Mseek Protocol
Developed by Jayaprakash et al. (2015), the Mseek protocol was designed for optimal mitochondrial and minimal nuclear DNA output. This was accomplished by digesting linear DNA with Exonuclease V, an enzyme developed by New England Biolabs.

mtDNA primers nuDNA primers

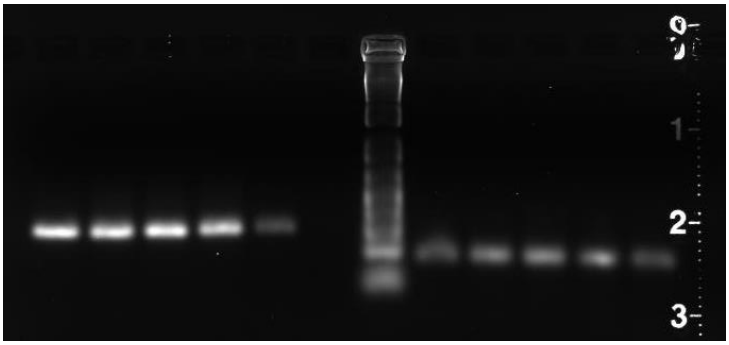
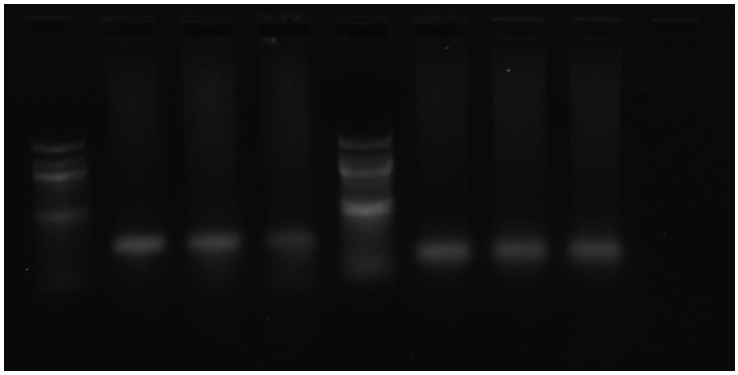


Figure 5: Amplified DNA After Extraction
After extraction, 10 samples containing equal volumes of DNA were separated into two groups- one was amplified with mitochondrial primers, and the other was amplified with nuclear primers. Between the two sets of samples is a DNA ladder.

mtDNA primers nuDNA primers

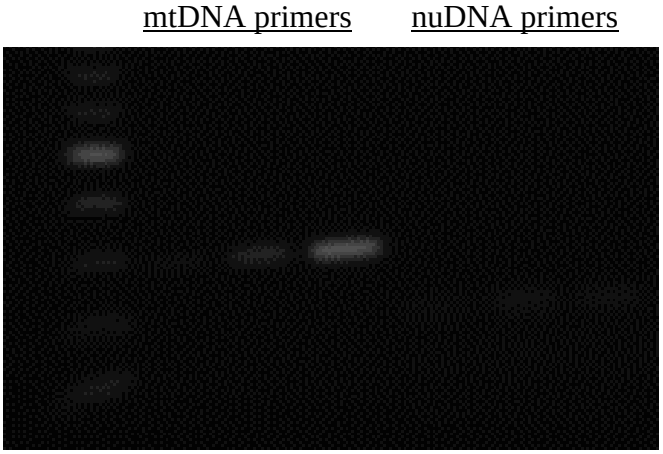


1 2 3 1 2 3

mtDNA
1. 4000 ng
2. 6000 ng
3. 8000 ng

nuDNA
1. 4000 ng
2. 6000 ng
3. 8000 ng

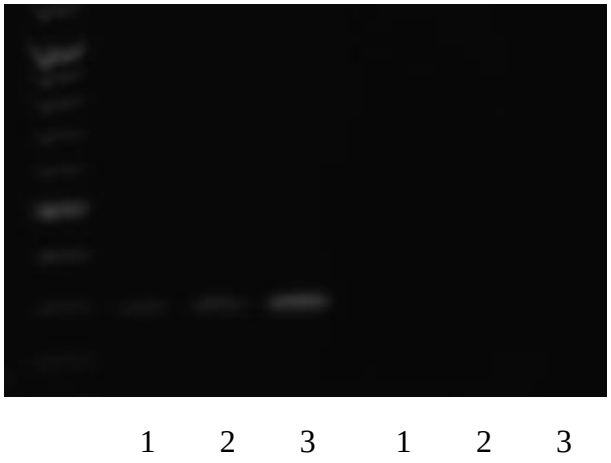
Figure 6: Amplified DNA After First Digestion with Exonuclease V
Samples were diluted to different DNA volumes and treated with Exonuclease V. Amplification was performed on samples of each volume with nuclear and mitochondrial primers.



mtDNA
1. 4000 ng
2. 6000 ng
3. 8000 ng

nuDNA
1. 4000 ng
2. 6000 ng
3. 8000 ng

Figure 7: Amplified DNA After First Digestion and First Purification
After treatment with Exonuclease V, purification was performed. PCR was performed with nuclear and mitochondrial primers to assess the effectiveness of the protocol.



mtDNA
1. 4000 ng
2. 6000 ng
3. 8000 ng

nuDNA
1. 4000 ng
2. 6000 ng
3. 8000 ng

Figure 8: Amplified DNA After Second Digestion and Second Purification (Final Product)
To determine success of the Mseek method, amplification was performed on the treated samples.

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