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Historical Genetic Analysis Of Horned Lark (Eremophila Alpestris) Specimens From The Prairie Pothole Region

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HISTORICAL GENETIC ANALYSIS OF HORNED LARK (*EREMOPHILA ALPESTRIS*) SPECIMENS FROM THE PRAIRIE POTHOLE REGION

by

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This thesis, submitted by Gregory Cain in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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This thesis is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

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Dean, School of Graduate Studies

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PERMISSION

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ABSTRACT

The Horned Lark (*Eremphila alpestris*) is a generalist bird species with a global distribution. A previous phylogeographic study of this species from western North America has been completed and identified three distinct mitochondrial DNA clades. This study aims to genetically characterize Horned Larks from the North Dakota region using the mtDNA NADH dehydrogenase subunit 2 (ND2) gene. Horned Larks were sampled from museum collections, with ages ranging from 13 - 120 years old. 5mm x 1mm feather cuttings were removed using a non-invasive feather sampling method from each specimen for genetic analysis.

This sampling method was verified on 15 total museum specimens of Horned Larks, Red-winged Blackbirds (*Agelaius phoeniceus*) and Franklin's Gulls (*Leucophaeus pipixcan*) from the University of North Dakota Vertebrate Museum Collection. Sampling methodology was investigated by using three mechanisms of DNA isolation: EDTA with proteinase K followed by column-based purification, chelating resin with dithiothreitol, and a Direct PCR method without DNA purification. Three fragments of mitochondrial DNA loci were amplified including control region, cytochrome c oxidase I and NADH dehydrogenase subunit 2. Correct sequencing results indicated that this method is viable for amplification of museum DNA, while at the same time limiting physical damage to the museum specimen. Arsenic found in low concentrations from arsenic test strips, was not a factor seriously hindering the success of PCR.

Phylogenetic reconstruction of historic Horned Lark genetic information using Bayesian Inference and Maximum Likelihood algorithms revealed the existence of a Nearctic South clade, and reclassifies the previously established Pacific Northwest clade

as a broader Nearctic North clade. The use of sequences with uneven preservation from museum specimens had no effect on overall tree topology. Horned Larks from the North Dakota region population had higher nucleotide diversity than other populations, and were all part of the Nearctic Northern clade. Five new haplotypes and three new SNPs were identified from museum specimens in this study.

CHAPTER 1
APPLICATION OF HISTORICAL AVIAN GENETICS (LITERATURE REVIEW)

DNA from Avian Museum Specimens

History of Avian Museum specimen Genetics

Since the day of Carl Linnaeus, natural history collections have provided information about the physical traits of organisms. This is especially true in the case of bird species (Winker, 2005). Avian museum specimens have also been used to obtain genetic information about past populations, which has led to greater understanding of the genetics of endangered and extinct species. The ability to extract and amplify historical DNA from avian museum specimens has been well established (Cooper 1994; Cooper et al., 2001; Shapiro et al.,2001; Kirchman et al., 2010). Museum specimens can also provide valuable insights into the population structure of a species on a temporal scale (Wandeler et al., 2007). Historical , less than 200 year old mitochondrial DNA (mtDNA) sequences have been retrieved from many species of North American bird museum specimens, including extinct species such as the Ivory-billed Woodpecker (*Campephilus principalis*) (Fleischer et al. 2006) and the Passenger Pigeon (*Ectopistes migratorius)* (Johnson et al. 2010). Small sequences of mitochondrial DNA (mtDNA) from persevered museum specimens have helped researchers resolve the taxonomic position and evolutionary relationships of many bird species from preserved museum specimens, including several extinct Hawaiian bird species (Fleischer et al., 2000). The Greater Prairie-Chicken (*Tympanuchus cupido*) (Johnson et al., 2004) was studied using preserved specimens to investigate genetic changes due to habitat fragmentation;

specimens of the Peregrine Falcon (*Falco peregrinus*) (Brown et al., 2007) were utilized to examine genetic structure pre-and post-population bottleneck threatened from DDT (dichlorodiphenyltrichloroethane) exposure.

Often the focus of historical avian DNA research is on extinct and endangered species found in large collections from national natural history museums. These museums are of great convenience to researchers as they can sample many individuals from a single repository (Wandeler et al,. 2007). However, it is unlikely that national museums will have specimens from all specific geographic locations and discrete time intervals of interest to researchers. Local collections such as university teaching and research collections, specimens collected by wildlife agencies and those held in private collections offer additional opportunities for historical investigation. These collections also may have the benefit of being physically close to points where specimens are collected. Local university museum collections may not have full time curatorial staff, and do not participate in organized collection trips aiming to obtain specific rare species. Specimens in local museums are often gathered over time by ornithology students and an environmentally aware public. These collections play important roles in small scale public education, and training future ornithologists, and could be used to increase sample size for systemic and population genetic based studies (Funk and Omland, 2003). One of the challenges in working with museum specimens from any collection is the quality of preservation techniques.

Specimen Preservation Methods

In the mid-18th century museum curators used salts, herbs, alum, spices or even tobacco to preserve specimens. These products were limited in their effectiveness, and after a short time the specimens began to decay (Marte et al., 2006). Arsenic soap, developed in the late 1700's , solved the problem of DNA decay. It was composed of camphor, arsenic oxide, carbonate of potash, soap and lime powder. In the 1980's arsenic soap was barred from use by the museum community because of its toxicity to humans (Le Dmiet and Jullien, 2002).

New methods of preservation have since been developed and are currently employed by many museums. These methods include complete dehydration of the specimen, initial freezing then storage in climate controlled facilities, and the use of non-toxic preservative chemicals. The preservation methods used on individual specimens could have an effect on the longevity of their DNA. Arsenic, a major preservation agent previously used in museum collections may inhibit DNA polymerase performance in PCR amplification (Töpfer et al., 2011).

DNA Sampling Methods

As a museum specimen ages the DNA within begins to break down into small fragments. Fragmented DNA becomes chemically modified and cross-linked. DNA and other organic compounds can form Maillard products that are sugar-rich tangles of proteins and nucleic acids. These factors often limit the amount of DNA that is retrievable from a museum specimen. The small yield of extracted DNA complicates and limits downstream applications of this historical material. Problems may results from PCR reactions because of low template copy numbers, or highly fragmented templates. Both of these factors promote jumping polymerase activity, which can result in a high

probability of nucleotide misincorporations during PCR.

Molecular techniques provide researchers with the ability to amplify DNA from physically small amounts of biological material. This permits genetic analysis of rare museum specimens, from which there is often a limited amount of valuable material from which to amplify DNA (Kirchman et al., 2010). While some studies have obtained amplifiable DNA from toe pads (Johnson et al., 2004; Fleischer et al., 2006; Brown et al., 2007; Johnson et al. 2010; Kirchman et al., 2010), feathers barbs (Speller et al., 2011), and the distal rachis of feathers (Rawlence et al., 2009), the use of these areas have the potential to damage taxonomically important characteristics. Preserved blood clots provide a sampling opportunity that does not cause damage to avian museum specimens (Horváth et al., 2005). However, the use of preserved blood clots when working with mtDNA could potentially lead to amplification of nuclear copies of mtDNA (numts) (Sorenson and Quinn 1998). Numts are copies of fragments of mtDNA that have been incorporated into nuclear DNA. These copies can change at a much faster rate than their mtDNA counterparts. If a numt has been incorporated in recent evolutionary time, enough mutations may not have accumulated within the sequences to prevent them from being amplified by primers for a PCR reaction targeting an mtDNA counterpart. Numts are a potential pitfall when working with historical mtDNA because without careful screening they can be misinterpreted as target DNA sequences. Unintentional use of numts sequences, which evolve differently from mtDNA and drastically inflate any measure of genetic diversity.

Avian mtDNA

Characteristics of the mtDNA genome

The avian mitochondrial genome (16,000-17,000bp) is circular, inherited maternally, and is assumed to be non-recombinant (Figure 1). It is composed of the three groups of genes: 22 tRNAs, 13 protein-coding regions, 2rRNAs and in most species one large noncoding region referred to as the control region or D-loop. The control region in birds can vary in sizes from 1072 bp in the Dunlin (*Calidris alpina*) to about 1240 bp in the Greenfinch (*Carduelis ambigua*) (Mindell, 1997). The first mtDNA genome sequenced for a bird species was the domestic chicken (*Gallus gallus domestics*). In this species a different order in which the NADH dehydrogenase 6 (ND6) and Cytochrome B(Cyt B) genes have changed location, this differed from than any other species at the time (Figure 1). Studies of avian mtDNA genomes indicate that this pattern of mtDNA genes happens across most of Class Aves including species such as: Quail, *Coturnix japonica* (Desjardins et al., 1991); Goose, *Anser sp.* (Quinn and Wilson, 1993); Dunlin, *Calidris alpine* (Wenink et al.,1993); Turnstone, *Arenaria interpres* (Wenink et al., 1994); Murres, *Uria sp.* (Moum and Johansen, 1992); subfamily Alcinae (Moum et al., 1994). Currently there are over 130 complete mtDNA genome sequences for species in Class Aves in the NCBI GenBank database.

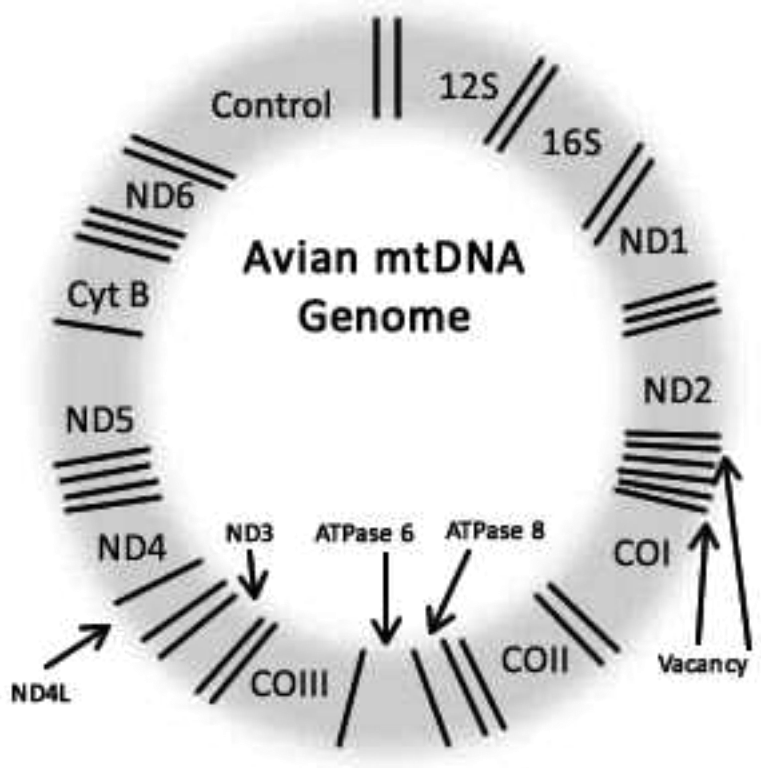

Figure 1. MtDNA gene order of the standard Avian mtDNA genome (*Gallus gallus domesticus*) (Desjardins and Morais, 1990). This figure was modified from Mindell et al. 1997.

Though the order of genes shown above is commonly found across birds, three alternative gene arrangements are known in various bird species (Eberhard et al., 2001; Abbott et al., 2005) (Figure 2). Gene rearrangements occur more frequently around the control region, because it contains the sequences for initiation and termination of replication and a clover structure on each end that could facilitate gene duplication and deletion events (Fujita et al., 2007). These gene rearrangements occur between the Cytochrome B gene and the 12S ribosomal RNA gene, and can include a duplication of the control region and surrounding genes (Verkuil et al., 2010).

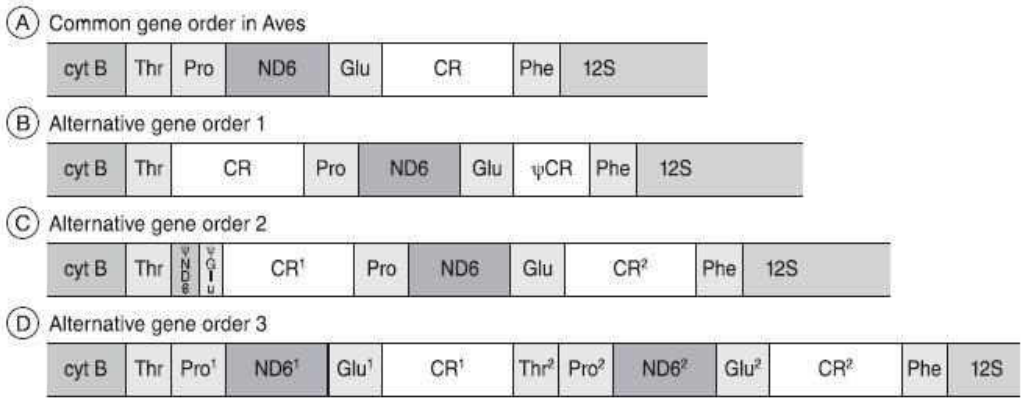

Figure 2. This figure was illustrated in Verkuil et al.(2010) and shows the four known gene orders in class Aves. (A) The chicken genome gene order (Desjardins and Morais, 1900). (B) The genome order in Passeriformes (Mindell et al., 1998; Bensch and Harlid, 2000). (C) The gene order in parrots (Eberhand et al., 2001). (D) The gene order in albatrosses (Abbot et al., 2005).

While the mitochondria is inherited as a linked set of alleles, the control region of mtDNA is commonly used in population genetics studies because of its relatively high rate of mutations. Control region sequence divergence ranges from about 12% to 25% within a species(Mindell 1997). The control region in birds is composed of two hypervariable regions (1 and 3) and a conserved region between the two (region 2). Gene rearrangements occur more frequently around the control region because it has the sequence for the initiation and termination of replication and a clover structure on each end that could facilitate gene duplication and deletion events (Fujita et al., 2007). These gene rearrangements occur between the Cytochrome B gene and the 12S (small ribosomal subunit) gene, and can include a duplication of the control region and surrounding genes (Verkuil et al., 2010). Three Alternative gene arrangements are found in various species such as parrots (Eberhard et al., 2001) and albatrosses (Abbott et al., 2005) (Figure 2).

Aside from the control region domains, the ND2 gene is the third most variable gene in the mitochondrial genome, and is commonly used in bird population genetic studies. Variation in the ND2 gene is evenly distributed across the whole gene, producing many haplotypes and reducing the probability of multiple substitutions at a single position because of the physical structure of the gene (Drovetski et al., 2005). This makes ND2 a better choice than the control region for use in population level especially if the species has not been investigated for the case of a control region duplication event. Historic DNA based studies of avian mtDNA should use the ND2 gene unless the full mtDNA genome of the bird species has been sequenced to insure that control region duplication events are not occurring in the studied species.

The Horned Lark

The Horned Lark (*Eremophila alpestris* L.) (order: Passeriformes family: Aludidae) is a an obligatory grassland monogamous bird species with migratory and resident individuals, and is protected under the Migratory Bird Treaty Act of 1918 (Figure 3). According to breeding bird survey data collected by the United States Geological Survey (USGS), the Horned Lark along with many bird species has shown a decline in annual abundance over the past 40 years (Sauer, 2011). A genetic study of Horned Lark populations in Western North America has confirmed the existence of a genetically distinct subspecies, based on the occurrence of a single haplotype of the mtDNA ND2 gene for all individuals identified as Streaked Horned Larks (*Eremphila alpestris strigata*)) (Drovetski et al., 2005). This subspecies is now listed as proposed for protection under the Endangered Species Act.

Figure 3. Photo of a Horned Lark (From U.S. Fish and Wildlife Service/ Willapa National Wildlife Refuge, WA).

The Horned Lark is widely distributed across North America and Asia, yet is the only lark species native to North America (Beason 1995). All other species of the lark family are native to Africa, Asia, and Europe, which makes the Horned Lark a unique species to study. A recent genetic survey of Horned Larks in the western United States found 30 unique haplotypes of the mtDNA ND2 gene in 99 individuals. Haplotypes were based on 43 substitutions (39 transitions and 4 transversions) at 42 variable sites (Drovetski et al., 2005). These substitution patterns formed the basis for reconstruction ofdistinct clade groups based on geographic location and genetics. Western United States Horned Lark genetic structure consists of three geographical clades, including: the Pacific Northwest (alpine and eastern Washington, Alaska), the Pacific Coast (western Washington, California), and the Great Basin (eastern Oregon) (Drovetski et al. 2005). AMOVA results also showed significant structuring of haplotypes among the three

clades. Differences among individuals within localities accounted for 12.2% of the sequence variation, while 75.7% of observed sequence variation was accounted for by clade differences. (Drovetski et al. 2005). These data indicate a high degree of mtDNA differentiation among clades and among some localities.

Recent research from China has shown 20 haplotypes ND2 for 50 individuals identified by 22 variable sites from a population on the Qinghai-Tibetan plateau (Qu et al., 2010). This population was found to have a nucleotide diversity of 0.0014 (Qu et al., 2010). This is a higher level of diversity found in all populations of Horned Larks North America (Table 1) except those dominated by the Streaked Horned Lark (Populations WAa,WAc, WAo,WAi in Table1) and the Alaskan interior population (Drovetski et al. 2005).

Table 1. Population, sample sizes, nucleotide diversity (π_n), and its standard deviation (sd) for each locality. Population Location Definitions: AKi – interior AK; AKc –Bering Sea coast, AK; WAo – vicinity of Olympia, WA; WAc – Pacific coast of WA; WAi – White island, WA; WAe – eastern WA; WAa – alpine tundra, Cascade mountains, WA.

Population	N	π_n	sd
Aki	18	0.001070	0.001027
AKc	5	0.001927	0.001794
WAe	16	0.005539	0.003442
WAa	4	0.000	N/A
OR	5	0.007322	0.005168
CA	19	0.003178	0.002186
WAc	5	0.000	N/A
WAo	21	0.000	N/A
WAi	5	0.000	N/A

Population structure in North American Horned Larks is possibly a result of glacial retreats following the last Ice Age (Drovetski et al., 2005). The use of glacial refugia by Horned Larks and its possible effects on contemporary genetic structure of a

population is supported by research from China based on phylogenetic reconstruction and population modeling (Qu et al., 2010).

Horned Larks from North Dakota region have been collected over the past 120 years and preserved by ornithology students and stored at the University of North Dakota and other nearby local university museums. These specimens were collected in the prairie pothole region, an extensive prairie grassland with small shallow wetlands dotted across the landscape, and provides a unique opportunity to study historic grassland bird population genetic diversity at a local scale in this habitat.

Objectives of this Study

Understanding how a species' genetic diversity varies across its geographic range and time is important, especially given current declines in optimal wildlife habitat. For many wildlife species, large databases of genetic information collected from multiple locations simply do not exist. Genetic data for wildlife species is even more temporally limited to recent time. Without the use of genetic material from museum specimens, only a limited understanding of how the genetics of an individual species has changed over time and space can be achieved. For example, by using only contemporary samples, a researcher would be unable to detect haplotypes that have not persisted though time to the present day population. The goal of this study is to investigate the genetic relationship of the historical Horned Lark North Dakota regional population to contemporary populations found in North America. To achieve this overall goal, the objectives of this study are to:

1) Collect a sample of 20 - 40 Horned Lark museum specimens from North Dakota archived in local museum collections.

2) Develop a method of sampling from avian museum specimens that ensures only minimal material not taxonomically important is destroyed. This method must be able to produce amplifiable mtDNA for sequencing, and be able to be repeated for assurance of quality control.

3) Conduct a PCR-based analysis of the mtDNA ND2 gene in an effort to understand the historical DNA diversity of the Horned Lark.

4) Compare the genetic diversity of historic North Dakota Horned Larks to that of current populations.

Study Hypotheses

In this study two hypotheses will be tested:

1) Museum specimens from local museum collections can provide DNA that is readily amplifiable for historic genetic analysis. This hypothesis will be explored in chapter 2.

2) Genetic diversity found in the historic population of Horned Larks from North Dakota is higher than in current populations found in North America. This hypothesis will be explored in chapter 3.

CHAPTER 2
METHODS

Museum Specimen Sampling

Prior to work with the Horned Lark, I performed a pilot study to assess the best methodology for proceeding with archival avian specimens. An initial sample of fifteen museum specimens of three small bird species were collected from the vertebrate museum at the University of North Dakota to investigate the viability of my sample method versus body size and other specimen traits. Three species from different families and of different body sizes were used to confirm that ability to recover DNA with this method was not unique to a single species.

Data including museum specimen numbers, location, and year collected were documented for Horned Larks (*Eremophila alpestris*), Red-winged Blackbirds (*Agelaius phoeniceus*), and Franklin's Gulls (*Leucophaeus pipixcan*) (Table 1). Sterile scalpels were used to make a 5 mm x 2 mm incision into the lower proximal rachis of a primary flight feather. Sampling from a primary flight feather's lower proximal rachis caused no damage to taxonomically informative characteristics, even after multiple samples had been collected (Figure 4). Two feather cuttings for each DNA isolation method, from each specimen, were stored in sterile 1.5ml tubes until DNA extraction.

Table 2. Specimens from the University of North Dakota (UND) Vertebrate Museum Collection used for sampling experiment.

Species	Lab Name	Location Collected	Year Collected	Museum ID
Franklin's Gull (*Leucophaeus pipixcan*)	FG1	Stump Lake, ND	1905	UND-AM-492
Franklin's Gull (*Leucophaeus pipixcan*)	FG2	Assumed from ND	1978	UND-AM-1207
Horned Lark (*Eremophila alpestris*)	HL1	Grand Forks ND	1979	UND-AM-1230
Horned Lark (*Eremophila alpestris*)	HL2	Grand Forks, ND	1966	UND-AM-711
Horned Lark (*Eremophila alpestris*)	HL3	Grand Forks, ND	1892	UND-AM-699
Horned Lark (*Eremophila alpestris*)	HL4	Towner, ND	1975	UND-AM-713
Horned Lark (*Eremophila alpestris*)	HL6	Kramer, ND	1975	UND-AM-712
Horned Lark (*Eremophila alpestris*)	HL7	Hoopie, ND	1975	UND-AM-714
Horned Lark (*Eremophila alpestris*)	HL8	Grand Forks, ND	1892	UND-AM-698
Horned Lark (*Eremophila alpestris*)	HL9	Grand Forks, ND	1892	UND-AM-701
Horned Lark (*Eremophila alpestris*)	HL10	Grand Forks, ND	1892	UND-AM-704
Red-winged Blackbird (*Agelaius phoeniceus*)	RW10	Upham, ND	1973	UND-AM-972
Red-winged Blackbird (*Agelaius phoeniceus*)	RW11	Embden, ND	1978	UND-AM-1206
Red-winged Blackbird (*Agelaius phoeniceus*)	RW12	Kelly's Slough, ND	1991	UND-AM-1402
Red-winged Blackbird (*Agelaius phoeniceus*)	RW9	Carrington, ND	1977	UND-AM-1231

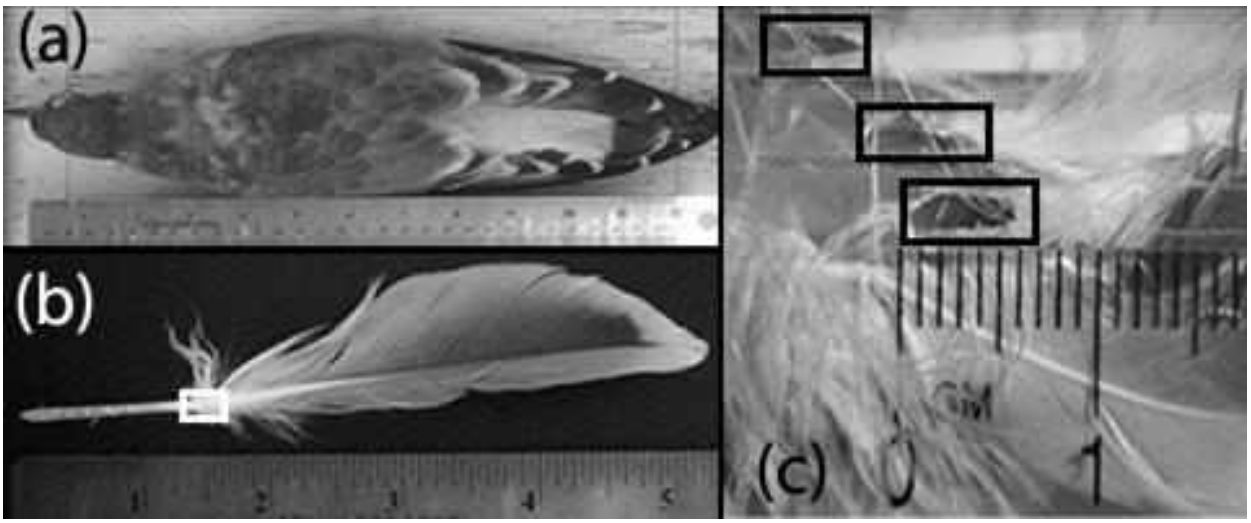

Figure 4. The non-invasive sampling method used to obtain DNA from the museum specimens in this study. This specimen is a Franklin's Gull collected in 1905 from Stump Lake, ND. (a) Full body of the Franklin's Gull specimen after sampling. (b) Approximate location of sampling from a feather removed from this specimen. (c) Three feather cuttings removed from this specimen without removal of feathers

To assesses Horned Lark genetic diversity in the North Dakota Region nineteen Horned Lark museum specimens ranging in age of 120 to 13 year old, were sampled from local collections in the Prairie Pothole region (twelve from the University of North

Dakota, five from North Dakota State University, two from the University of Minnesota Crookston) (Table 3). To limit damage to museum specimens, the previously described non-invasive sampling technique was employed in the collection of material for genetic analysis.

Table 3. Year and Location collected of Horned Lark museum specimens used in determining historical genetic diversity of the North Dakota Region

Year Collected	Location	Museum Specimen Number
1892	Grand Forks, ND	UND-AM-699
1892	Mountain Trail, ND	UND-AM-700
1892	Grand Forks, ND	UND-AM-698
1892	Grand Forks, ND	UND-AM-701
1892	Safton, ND	UND-AM-709
1915	Fargo, ND	NDSU-496
1966	Grand Forks, ND	UND-AM-711
1966	Valley City, ND	NDSU-2298
1966	Fargo, ND	NDSU-2255
1966	Belfield, ND	NDSU-2137
1966	Belfield, ND	NDSU-2136
1975	Towner, ND	UND-AM-713
1975	Kramer, ND	UND-AM-712
1975	Hoopie, ND	UND-AM-714
1979	Grand Forks, ND	UND-AM-1230
1982	Grand Forks, ND	UND-AM-704
1982	Grand Forks, ND	UND-AM-702
1998	Gentilly, MN	UMC-492
1999	Crookston, MN	UMC-493

Arsenic Testing

Arsenic was used as a preservation agent for museum specimens at the University of North Dakota vertebrate museum (Seabloom, pers. comm.). To assess how much of an impact this preservation agent may have on DNA amplification from these museum specimens in this collection, an Arsenic Paper Test Kit (Macherey-Nagel Corporation) was used to estimate the arsenic concentration in three areas including sampling area

(feather), head, and toe pad. Test strips were visually compared to arsenic concentration controls from a nine-step 1:2 serial dilution of available 3.627 mg/L sodium arsenate stock solution.

DNA Extraction

All pre-PCR DNA work was completed in an isolated ancient DNA laboratory (Ovchinnikov Laboratory) at the University of North Dakota following stringent protocols to avoid contamination, including daily bleaching and the use of UV-Lights to limit the possibility of foreign and between sample contamination (Wandeler et al. 2007). In the sampling experiment, two feather cuttings from each specimen were used in separate DNA extractions for all three methods, whereas in the Horned Lark genetic diversity assessment DNA was extracted from a single feather cutting.

The first DNA isolation method tested on feather cuttings was an EDTA (Ethylenediaminetetraacetic acid) based method (ZR Genomic DNA Tissue Micro Prepararion, Zymo Research) following the manufactures protocol for DNA extraction from solid tissue. 90µl DNA free water 90µl 2x Digestion Buffer and 10 µl Proteinase K were added to each feather cutting. Samples were then vortexed and incubated at 55° C for 3 hours in a digital dry bath . 700µl Genomic Lysis Buffer was added to the tube, vortexed, centrifuged at 10,000 x g for one minute to remove insoluble debris. The supernatant was transferred to a Zymo-Spin Column in a Collection Tube, centrifuged at 10,000 x g for one minute. Next 200 µl of DNA Pre-Wash Buffer was added to the spin

column in the collection tube and centrifuged at 10,000 x g for one minute. 400 µl of g-DNA Wash Buffer was then added to the spin column and centrifuge at 10,000 x g for one minute. The spin column was transferred to a clean 1.5ml tube. Finally 10ul DNA Elution Buffer was added, allowed to incubate 5 minutes at room temp, and then centrifuged at max speed for 30 sec.

The second DNA isolation method tested was a chelating resin (Chelex-100, Bio-Rad) with dithiothreitol (DTT) method. This method is commonly used in forensic laboratories as a low cost method to process samples for DNA isolation. Chelex -100 was prepared in a 10% concentration for long term storage at 4°C and DNA extraction followed a modified form of the protocol for DNA extraction from hair described by Walsh et al. (1991). 100 µl of 10% Chelex-100, 100µl of DNA free water, and 40µl of 2mg/ml DTT solution was added to each feather cutting, then incubated at 56°C overnight in a GeneMate digital dry bath (BioExpress). After incubation samples, were vortexed and incubated at 100°C for 15 minutes. Samples were then centrifuged for 2 minutes at 3500 x g. Supernatant was collected for PCR applications.

The final DNA isolation method used for the sampling experiment was the Direct PCR method (Terra PCR Direct, Clontech). Feather cuttings were added directly to aliquoted Terra Direct PCR mix. This 50µl reaction was composed of ;25µl of 2X Terra PCR Direct Buffer with Mg2+ and dNTP, 3µl of each forward and reverse primer for the target species (Appendix A Supplemental Table 1), 1µl of Terra PCR Direct Polymerase Mix, and 18µl of DNA free water. Forty PCR cycles were performed using the manufactures guidelines.

All DNA extracts, for the sampling experiment, were measured on a Nanodrop Spectrophotometer (Thermo Scientific, ND-1000) for DNA concentration and DNA extract purity to provide a basis for selection of a single DNA extraction method for use in determining Horned Lark genetic diversity. The Chelex-100 method was chosen to be used in the Horned Lark genetic diversity study. All DNA extractions contained at least one extraction blank to test for contamination, and were stored in sterile 1.5ml tubes at 20°C until used for PCR.

PCR and Sequencing

In the sample experiment, fragments of three commonly used mtDNA loci were used to test for amplifiable DNA. These fragments included a 181 amplicon of the ND2 mtDNA gene from Horned Lark specimen, a 330 bp amplicon of the control region from Franklin's Gull specimens, and a 224 bp amplicon of COI mtDNA gene from Red-wing Blackbird specimens using species specific primers (Appendix A Supplemental Table 1). For the genetic diversity study, the Horned Lark mtDNA ND2 gene was divided into overlapping sections of less than 200bp in size for primer design, including the greatest possible number of known single nucleotide polymorphisms (SNPs) from aligned published sequences in each section(Appendix A Supplemental Table 2).

PCR was carried out in a volume of 25µl including 10µl template DNA, 7.5 µl DNA Free PCR water (BioExpress), 5µl 5x Go Taq Flexi Buffer, 1.5µl MgCl, 0.3µl 5µM forward primer, 0.3µl 5µM reverse primer and GoTAQ DNA Polymerase (5 units/µl) (Promega). All PCR reactions contained at least one negative control to screen for contamination between samples; this was accomplished by including all reagents from a

PCR reaction with the exception of any template DNA. PCR was performed under the following conditions: 12 minutes initial denaturing step at 94°C, followed by 40 cycles at 94°C for 1 minute, primers were allowed to anneal for 1 min at a temperature specific for each primer pair, then normal extension 72° C for 1 minute, and a final extension step at 72°C for 8 minutes. All PCR products as well as negative extraction and PCR controls were visually inspected by gel electrophoresis in a separate post-PCR room. Positive PCR results were cleaned up with DNA Clean & Concentrator™- 5 (Zymo Research).

Only a subsection of positive PCR results were sequenced for the sampling experiment to confirm that mtDNA sequences matched intended target bird species and gene. In the Horned Lark genetic diversity study all positive PCR results were sequenced in the forward and reverse directions in an effort to cover as much of the ND2 gene as possible and screen out possible sequencing errors.

PCR Amplicon concentration was estimated using band intensity by referencing ladder concentration on the same agarose gel. Sequencing reactions were conducted using BigDye Terminator v3.1 Cycle Kit (Applied Biosystems). Sequencing reactions were composed of 2.37 µl SEQ(DNA Free) Water, 1.5µl 5 x Sequencing Buffer, 0.5µL BigDye RR Mix and 0.33 µl of a forward or reverse primer. The amount of SEQ (DNA Free) Water was adjusted to insure all reactions were 10 µl in volume based on concentration of amplicons from PCR reactions. Sequencing reactions were cleaned up using Bigdye Exterminator solution or ethanol precipitation. Sequencing reactions were separated on an Applied Biosystems 3100 Genetic Analyzer. Sequences were aligned and compared to those found in GenBank using BLAST (http://www.ncbi.nlm.nih.gov")

Statistical Methods

To understand what factor(s) may play an important role in determining PCR success, a GLM model was constructed using the following variables from the sampling experiment: bird species, age of museum specimen, and DNA extraction method. DNA extraction concentration (nanograms of DNA per microliter), DNA extraction purity (260/280 wavelength fluorescence ratio) were included to the model, but were averaged for each museum specimen.

The sample size for this experiment was small, thus unsuited for approaches used in large complex statistical analysis. A parsimonious approach, disregarding possible complex interactions between variables that require large sample numbers to compute accurately was used. The GLM was constructed using a binary response variable with multiple predictor variables (logistic regression). All assumptions such as colinearity, lack of fit, linear relationships and influence and dispersion of data were tested in R following the procedure outlined in Logan (2011).

Phylogenetic Methods and Population Comparisons

A database of 103 Horned Lark mtDNA ND2 gene sequences was constructed from available published sequences in the NCBI GenBank database (accession numbers AF290100.1, AF407049.1, DQ18738-DQ187487, EU307809.1) This database was composed of Horned Lark DNA from 11 populations: AKi – interior AK, AKc –Bering Sea coast, AK, WAo – vicinity of Olympia, WA, WAc – Pacific coast of WA, WAi – White island, WA, WAe – eastern WA, WAa – alpine tundra, Cascade mountains, WA, ,OR, CA. and single samples from Montana and Arizona. North Dakota region ND2

gene sequences from the genetic diversity study were added to make a twelfth population. Alignment was conducted using the ClustalW algorithm in MEGA5.01 (Tamura et al. 2007). The DNA model HKY+G was used for phylogenetic reconstruction based on model selection test results of the ND2 gene in MEGA 5.01. Maximum Likelihood phylogenetic reconstruction was performed in MEGA5.01 with 10,000 bootstrap replications. Bayesian Inference was performed using Mr. Bayes 3.1.2 (Huelsenbeck & Ronquist, 2001) run for 10,000,000 iterations were used for phylogenetic tree reconstruction. A closely related species, the Temminck's Lark (*Eremophila bilopha*) from Africa was used as an out group for all analyses. Population comparisons based on nucleotide diversity and pairwise F_{st} values were calculated in Arlequin 3.5 (Excoffier and Lischer 2010).

CHAPTER 3
RESULTS: THE UTILITY OF SMALL AVIAN MUSEUM SPECIMENS FROM LOCAL MUSEUM COLLECTIONS FOR HISTORIC GENETIC STUDIES

Spectrophotometer results indicate Chelex-100 with DTT isolation resulted in the most nanograms per micro liter of DNA (Table 4/Figure 5). It is important to note that a spectrophotometer measures the entire DNA in a sample. Since my work is on museum specimens, some of this DNA could be human, fungal or bacterial DNA , which might explain the high variance values of DNA concentration with the Chelex-100 method. The Chelex-100 method also exhibited the more pure DNA samples as defined by a ~1.8 value for the 260/280 ratio (Henry 1997). Using the Terra Direct PCR, it is impossible to quantify DNA extraction concentration and purity independent of the PCR reaction. Experiments using this method of DNA isolation were excluded from statistical analysis.

Table 4. Sampling experiment comparing Ng/µl and 260/280 ratios of the Chelx-100 and Zymo isolation methods.

Bird Sample	Chelex Ng/µl	Chelex 260/ 280 Ratio	Zymo Ng/µl	Zymo 260/280 Ratio
FG1 Feather Cutting 1	515.768	0.606	8.036	1.448
FG1 Feather Cutting 2	520.436	0.646	4.52	1.668
FG2 Feather Cutting 1	162.054	0.62	4.584	1.082
FG2 Feather Cutting 2	245.92	0.578	2.11	3.71
HL1 Feather Cutting 1	8.354	1.566	-0.082	3.412
HL1 Feather Cutting 2	400.26	1.422	0.974	1.23
HL2 Feather Cutting 1	324.92	2.104	2.554	1.474
HL2 Feather Cutting 2	470.512	1.048	2.014	0.926
HL3 Feather Cutting 1	502.866	1.162	1.366	2.806
HL3 Feather Cutting 2	310.972	0.8	0.292	0.336
HL4 Feather Cutting 1	436.51	1.466	0.446	0.462
HL4 Feather Cutting 2	523.534	0.83	0.2	-0.3
HL6 Feather Cutting 1	437.07	1.212	1.064	2.724
HL6 Feather Cutting 2	346.646	1.028	1.024	0.124
HL7 Feather Cutting 1	265.208	1.746	0.554	-0.348
HL7 Feather Cutting 2	424.822	1.778	-0.232	0.646
HL8 Feather Cutting 1	466.07	1.242	0.26	0.468
HL8 Feather Cutting 2	410.976	1.482	-0.39	-0.178
HL9 Feather Cutting 1	316.188	1.352	-0.038	3.874
HL9 Feather Cutting 2	466.43	0.82	0.814	0.53
HL10 Feather Cutting 1	378.592	1.852	-0.432	0.524
HL10 Feather Cutting 2	340.618	1.322	-0.022	1.208
RW9 Feather Cutting 1	133.796	0.916	-0.238	-0.008
RW9 Feather Cutting 2	663.446	1.234	0.948	0.528
RW10 Feather Cutting 1	345.64	1.22	-0.118	-0.348
RW10 Feather Cutting 2	304.11	0.978	3.214	1.33
RW11 Feather Cutting 1	256.918	0.764	-0.476	-0.656
RW11 Feather Cutting 2	374.192	1.828	0.542	0.502
RW12 Feather Cutting 1	518.936	1.03	-0.766	0.41
RW12 Feather Cutting 2	253.538	0.79	0.134	1.168

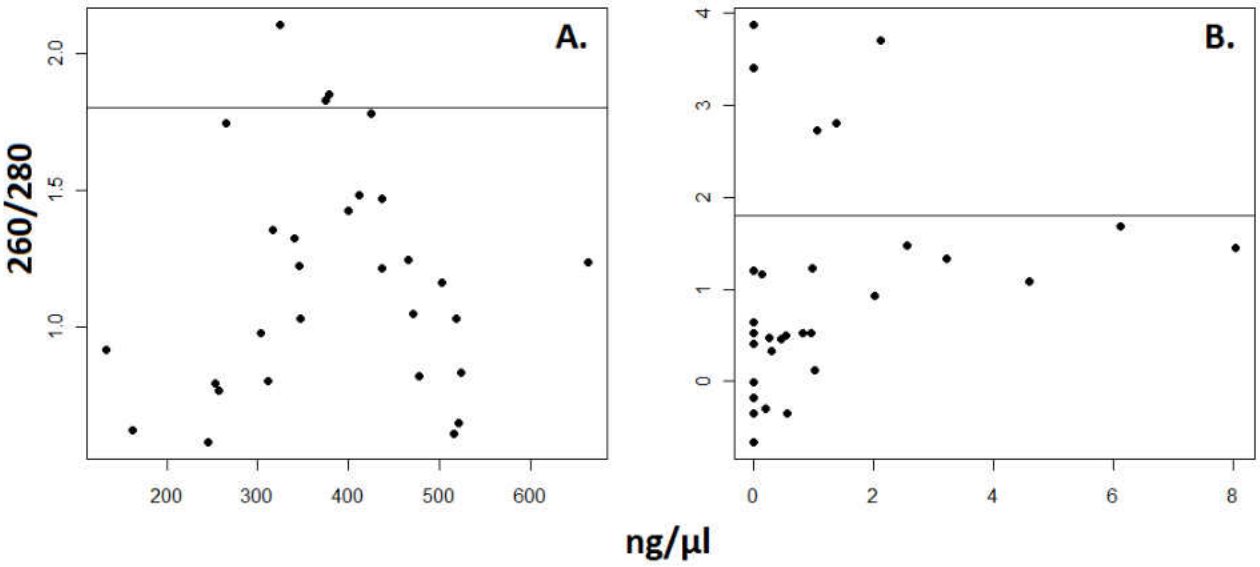

Figure 5. Comparison of DNA extract concentration and purity of the Chelex-100 (A) and the Zymo (B) isolation methods. DNA extraction concentration (ng/µl) is plotted vs. the DNA extraction purity (260/280 ratio) for each DNA extraction (n=30). The horizontal line on each graph displays the ~1.8 optimum 260/280 ratio.

All fifteen bird specimens had a total of six samples collected from them. Two independent DNA extractions were performed with each of the three DNA isolation methods. PCR was conducted on each of these independent DNA extractions. Successful amplification of PCR products from museum specimens ranged from 53.3% via Direct PCR to 60.0% with Chelex -100 and the Zymo Kit (Table 5). These results are consistent with those observed in similar DNA research (Martínez-Cruz et al. 2007, Rawlence et al. 2009). The PCR products had the proper size and DNA sequencing results confirmed that they originated from the correct species (Figure 6).

Table 5. PCR success with each DNA isolation method.

Age group ~100 years					
Species	Year	Size (bp)	Chelex	Zymo	Direct
Horned Lark	1892	181	Y	N	Y
Horned Lark	1892	181	N	N	N
Horned Lark	1892	181	N	N	N
Horned Lark	1892	181	N	N	N
Franklin's Gull	1905	330	Y	Y	N
Age group ~50 years					
Species	Year	Size(bp)	Chelex	Zymo	Direct
Horned Lark	1966	181	Y	Y	Y
Red-winged Blackbird	1973	224	N	N	Y
Horned Lark	1975	181	Y	N	Y
Horned Lark	1975	181	Y	Y	Y
Horned Lark	1975	181	Y	Y	Y
Age group ~25 years					
Species	Year	Size(bp)	Chelex	Zymo	Direct
Red-winged Blackbird	1977	224	N	Y	Y
Franklin's Gull	1978	330	N	Y	N
Red-winged Blackbird	1978	224	Y	Y	N
Horned Lark	1979	181	Y	Y	Y
Red-winged Blackbird	1991	224	Y	Y	N

Successful PCR reactions (Y) from at least one of two feather cuttings with each method and unsuccessful PCR reactions (N) from none of two feather cuttings are listed under the designated isolation method.

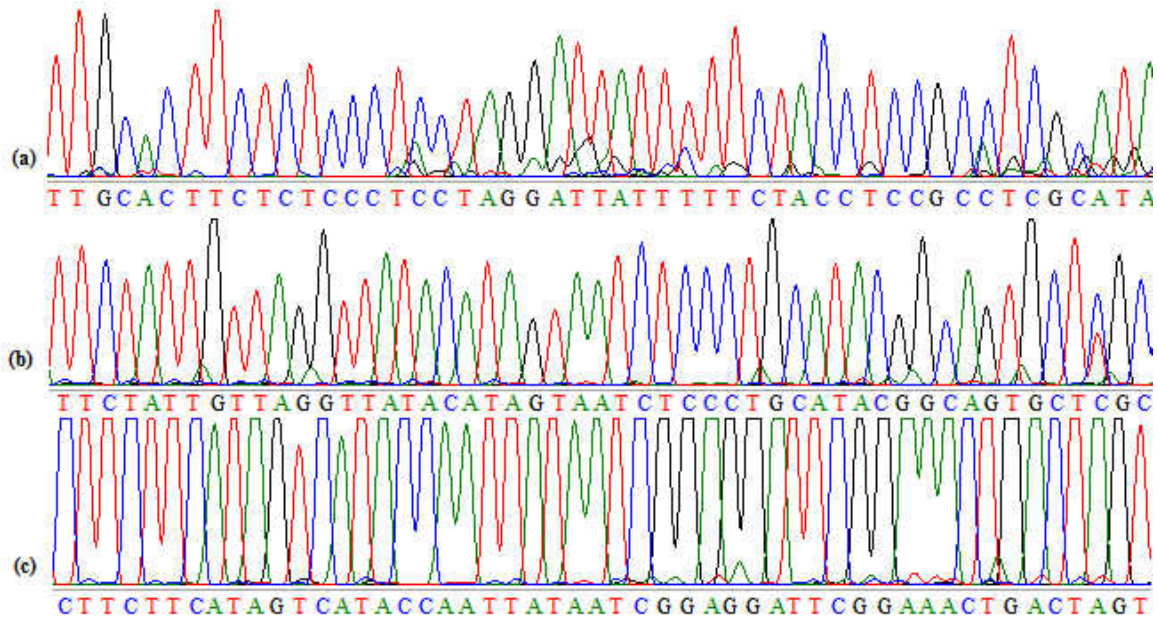

Figure 6. DNA sequence results from initial test of sampling method on UND museum collection specimens (a) Horned Lark isolated with Chelex-100. (b) Horned Lark isolated with Chelex-100. Franklin's Gull DNA isolated with the Zymo kit. (c) Red-winged Blackbird amplified with Direct PCR. Shown are 50 bp sections of sequenced PCR products as examples of quality results.

An approximate estimation of the arsenic concentration on specific areas of our museum specimens was made from a visual comparison of the arsenic test strips to color standards. The smallest amount detectable by this test is 0.02 mg/L. Concentration values ranged from <0.02 - <0.056 mg/L in our sampling area, <0.02 - <0.056 mg/L in heads sampled, and <0.02 - <0.227 mg/L in toe pads sampled. Arsenic was only detected in two Horned Lark specimens collected in 1892 and 1979. In these two birds, arsenic concentration levels were not uniform in all three areas sampled (Table 6). Arsenic concentration levels were far lower than the observed concentration of PCR inhibition 5400 mg/L (Töpfer et al. 2011a).

Table 6. Observed arsenic concentration values (mg/L) in UND museum specimens.

Museum Specimen	Sampling Area	Head	Toepad
HL1	<0.056	<0.056	<0.227
HL2	<0.02	<0.02	<0.02
HL3	<0.02	<0.02	<0.02
HL4	<0.02	<0.02	<0.02
HL6	<0.02	<0.02	<0.02
HL7	<0.02	<0.02	<0.02
HL8	<0.02	<0.02	<0.02
HL9	<0.02	<0.056	<0.02
HL10	<0.02	<0.02	<0.02
FG1	<0.02	<0.02	<0.02
FG2	<0.02	<0.02	<0.02
RW9	<0.02	<0.02	<0.02
RW10	<0.02	<0.02	<0.02
RW11	<0.02	<0.02	<0.02
RW12	<0.02	<0.02	<0.02

The GLM model produced one clear, best fit model with age as the only variable to explain the PCR success rate in our museum specimens (Figure 7). Full GLM modeling results are shown in Appendix B. All other models using combinations of other variables considered were deemed uninformative, because of how well age explained PCR success based on AICc (Akaike Information Criterion with correction for small sample size) score and model weight comparisons. An exact chi-square test for independence (test statistic 0.9842, 2-sided P-value 0.6633) performed in Cytel StatXact v9 concluded there was no difference on PCR success rate based on bird species (Figure 8).

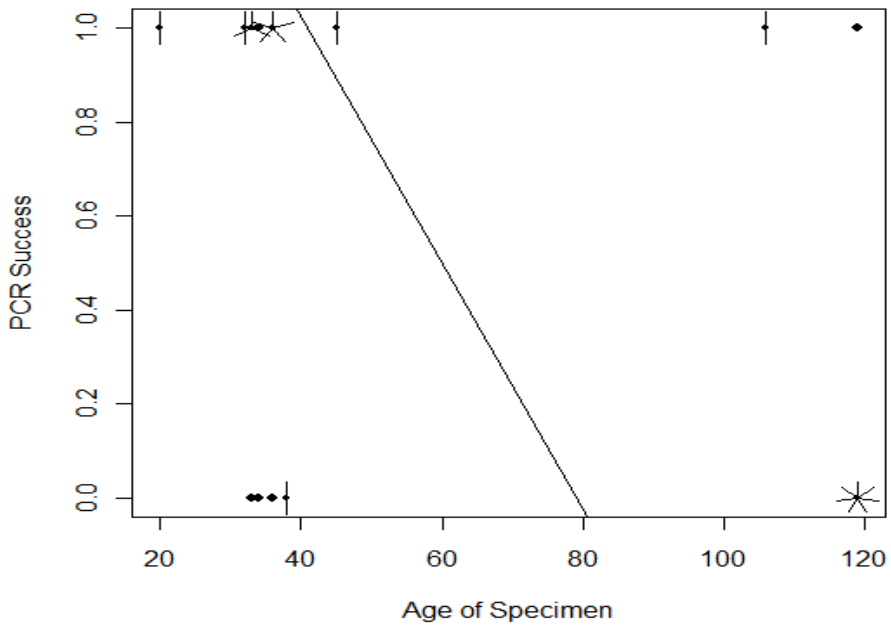

Figure 7. Sunflower scatter plot of the best fit model using age as binary predictor of PCR success with local museum specimens. (Intercept: 2.0730, Slope: -0.02620, Residual Deviance: 33.54, Null Deviance: 40.38). Multiple points in a single location are represented by the corresponding number of pedals originating from a single point.

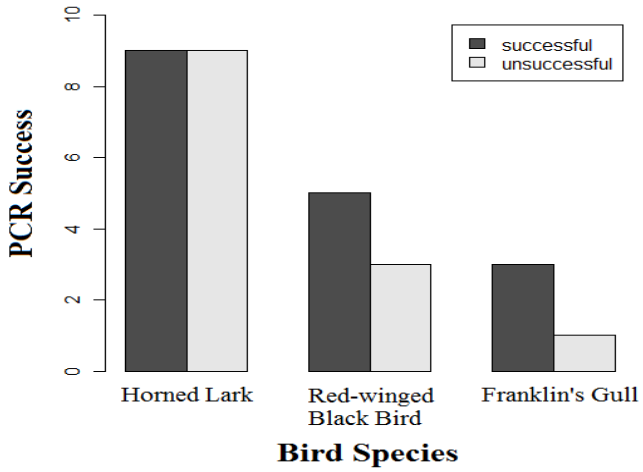

Figure 8. No statistical difference in rate of PCR success per species.

CHAPTER 4
RESULTS: NORTH DAKOTA REGIONAL HORNED LARK (*EREMPHILA ALPESTRIS)* POPULATION AND PHYLOGENETIC DIVERSITY ASSESSMENT

I found evidence of uneven DNA preservation in North Dakota avian museum specimens. Only one Horned Lark specimen failed to yield any amplifiable mtDNA. Within gene preservation varied greatly and only three of eight initially targeted sections of the ND2 gene produced consistent results (Table 7). The three noncontiguous sections of this gene totaling 519 bp produced consistent sequencing results that were used for analysis.

Table 7. Unequal preservation in Horned Lark mtDNA ND2 gene.

Horned Lark Sample	Section 2(110 - 300)	Section 5(460 - 607)	Section 7(722 - 903)
UND-AM-699	X	-	X
UND-AM-700	-	-	-
UND-AM-698	X	-	X
UND-AM-701	X	-	X
UND-AM-709	X	-	X
NDSU-496	X	X	-
UND-AM-711	X	X	X
NDSU-2298	X	X	X
NDSU-2255	X	X	X
NDSU-2137	X	X	X
NDSU-2136	X	X	X
UND-AM-713	X	X	X
UND-AM-712	X	X	X
UND-AM-714	X	-	X
UND-AM-1230	X	-	X
UND-AM-704	-	-	X
UND-AM-702	-	X	X
UMC-492	X	X	X
UMC-493	X	X	X

X indicates a sequence was produced for that bird and section of the ND2 gene, while a dash indicates no sequence result.

The North Dakota regional population of Horned Larks can be characterized by six haplotypes for eighteen individuals (Figure 9). The reference sequence I used to determine SNPs was Genbank Accession number DQ187487.1. I discovered three new SNPs (2 transitions, 1 transversion). The first, a C→T transition, was located at the 197 nucleotide position. The second, also a C→T transition, was found at the 240 nucleotide position in the ND2 gene. The final new SNP, a C→G transversion, was located at the 123 nucleotide position. Three previously established SNPs were found (all 3 transitions)within the North Dakota Regional population, including a T→C transition located at the 533 nucleotide position ,a T→C transition at the 555 nucleotide position, and a A→G transversion at the 822 nucleotide position.

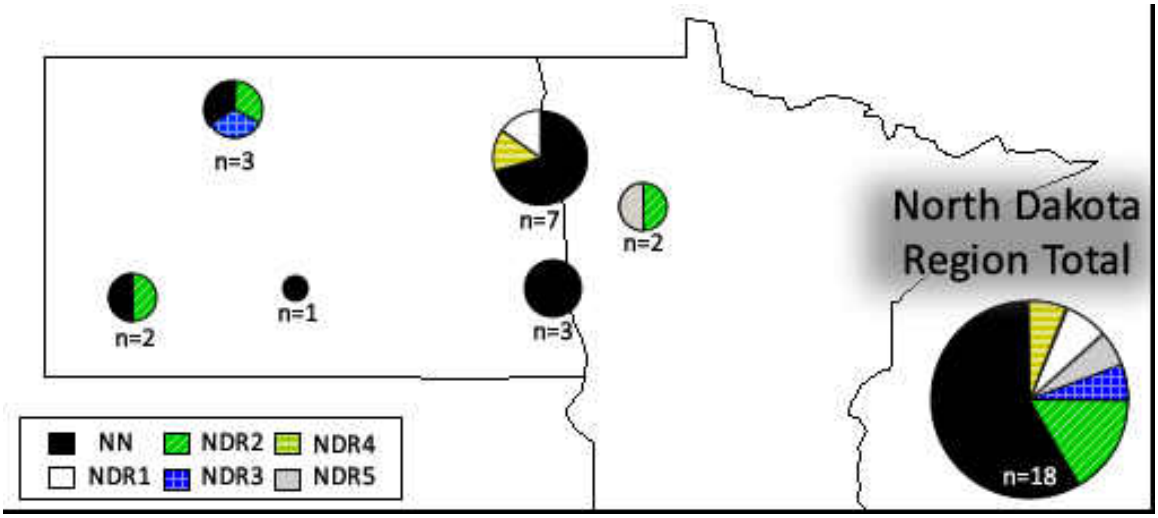

Figure 9. ND2 Horned Lark Haplotype distribution within the North Dakota regional population. Six haplotypes were found with the Nearctic North haplotype (NN) at the highest frequency across the landscape. North Dakota Region haplotypes are named in order of discovery.

A single silent amino acid change, caused by the novel transition at the 240 nucleotide C→T in the Horned Lark ND2 gene. All other SNPs found within the North Dakota population did not result in any amino acid changes (Table 8).

Table 8. ND2 haplotype characterization of the North Dakota Regional population.

Haplotype	**Number of Mutations**	**Location and Mutation**	**Novel ND2 Amino Acid Changes**
NN	N/A	None, Nearctic North Haplotype	N/A
NDR1	2	533bp T→C, 822bp A→G	0
NDR2	3	533bp T→C, 555bp T→C, 822bp A→G	0
NDR3	1	822bp A→G	0
NDR4	2	197bp C→T*, 240 C→T*	1, A→C
NDR5	1	123bp C→G*	0

* Indicates a novel SNP mutation in Horned Larks

New SNPs were observed in two birds. A single bird collected in 1892 contained two of these new SNPs, located at the nucleotide positions of 197 and 240 along the ND2

gene. The other bird, collected in 1998 had one new SNP located at the position of 123 nucleotide along the ND2 gene.

The North Dakota Regional population had higher nucleotide diversity (π_n = 0.0524 sd= 0.0271) when compared to other populations (Table 9). The Nearctic North (NN) haplotype is identified by Genbank Ascension number DQ187487.1.

Table 9. Population, sample sizes, nucleotide diversity (π_n), and its standard deviation (sd) for each locality.

Population	N	Clade	π_n	sd
NDR	18	Nearctic North	0.052379	0.027074
MT	1	Nearctic North	0.000	N/A
Aki	18	Nearctic North	0.001070	0.001027
AKc	5	Nearctic North	0.001927	0.001794
WAe	16	Nearctic North	0.005539	0.003442
WAa	4	Nearctic North	0.000	N/A
OR	5	Great Basin	0.007322	0.005168
CA	19	Pacific Coast	0.003178	0.002186
WAc	5	Pacific Coast	0.000	N/A
WAo	21	Pacific Coast	0.000	N/A
WAi	5	Pacific Coast	0.000	N/A
AZ	1	Nearctic South	0.000	N/A

Population Location Definitions: NDR - North Dakota Region; AKi – interior AK; AKc –Bering Sea coast, AK; WAo – vicinity of Olympia, WA; WAc – Pacific coast of WA; WAi – White island, WA; WAe – eastern WA; WAa – alpine tundra, Cascade mountains, WA

To find the usefulness of these sequences with gaps in sections a comparative method was employed. In all phylogenetic tree constructions, two datasets were used. One denoted “gap” in which specimens with observed uneven preservation were included in the dataset (n=122). This approach emphasizes obtaining the largest sample size possible, but not complete sequences for all individuals. The second, “without gap”, dataset (n=115) consisted of only specimens in which no uneven preservation was observed. This approach emphasizes more information about a smaller sample of the

historic population.

A phylogenetic tree that was constructed using 519 bp of the mtDNA ND2 gene produced four distinct clades in North America (Figure 10). Statistical support is listed for posterior probability above branch nodes. Bootstrap values are listed below branch nodes. The two different datasets, without gap and gap data sets, are separated by a | symbol respectively. The tree topology for all four phylogenetic reconstructions was the same, differing only by values of branch support. The Great Basin (0.96|0.94, 0.63|0.61) and Pacific Coast (0.99|0.60, 0.71|0.60) clade composition remained the same as previously published (Drovetski et al. 2005). The Pacific Northwest clade has been renamed Nearctic North (0.99|0.52,0.28|0.21), because all samples from the North Dakota region and one sample from Montana (Klicka et al. 2000) fell into this clade. A single sample from Arizona (Sorenson and Payne 2001) formed the Nearctic South clade (1|1, 0.08|0.09). Gap and without gap datasets produced the same overall tree topology, though the use of gap sequences reduced tree support (Figure 10). All four clades are geographically concordant (Figure 11). The Nearctic North (NN) haplotype identified by Genbank Ascension number DQ187487.1 is the most frequent haplotype in this clade, found in a polytomy in each of the four trees reconstructions (Figures 12-15).

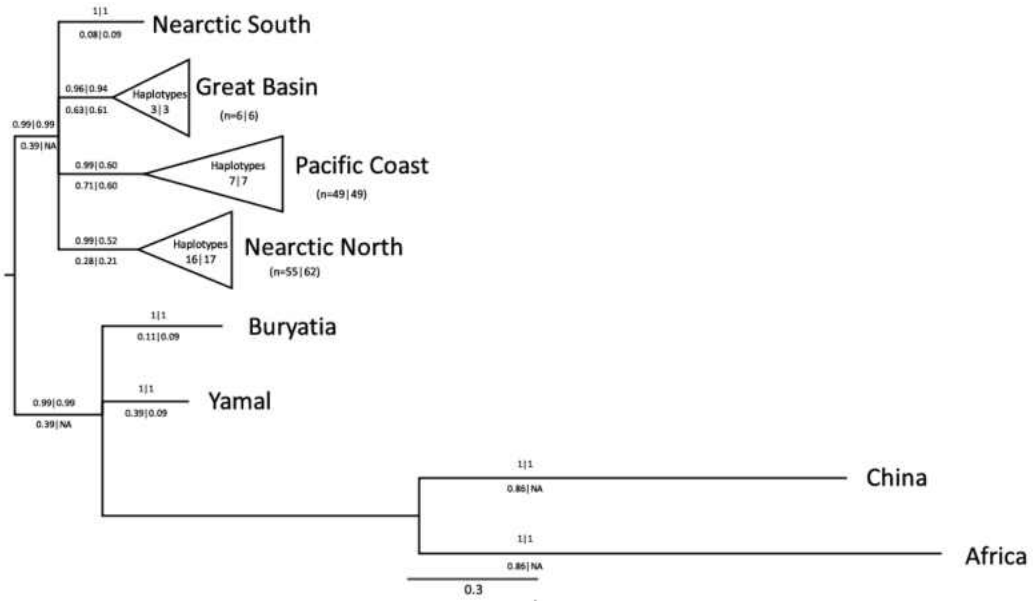

Figure 10. Global phylogenetic tree of genus *Eremophila.* Bayesian inference posterior probabilities based on 10,000,000 iterations are shown above branches, bootstrap values for Maximum Likelihood based on 10,000 replications are shown below branches. Values before | (vertical dash) are from the without gap datasets, while after | are from the uneven DNA preservation dataset.

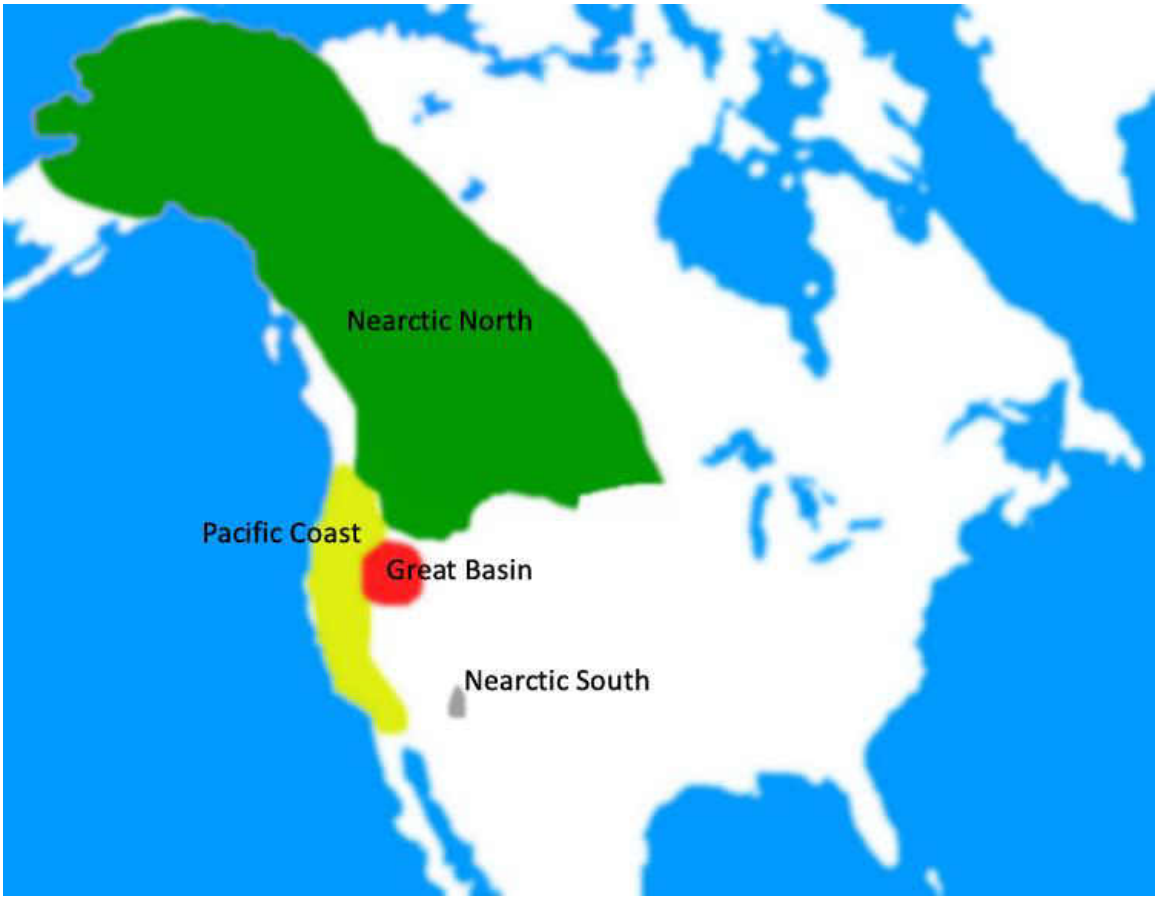

Figure 11. Phylogenetic clade distribution of Horned Larks in North America.

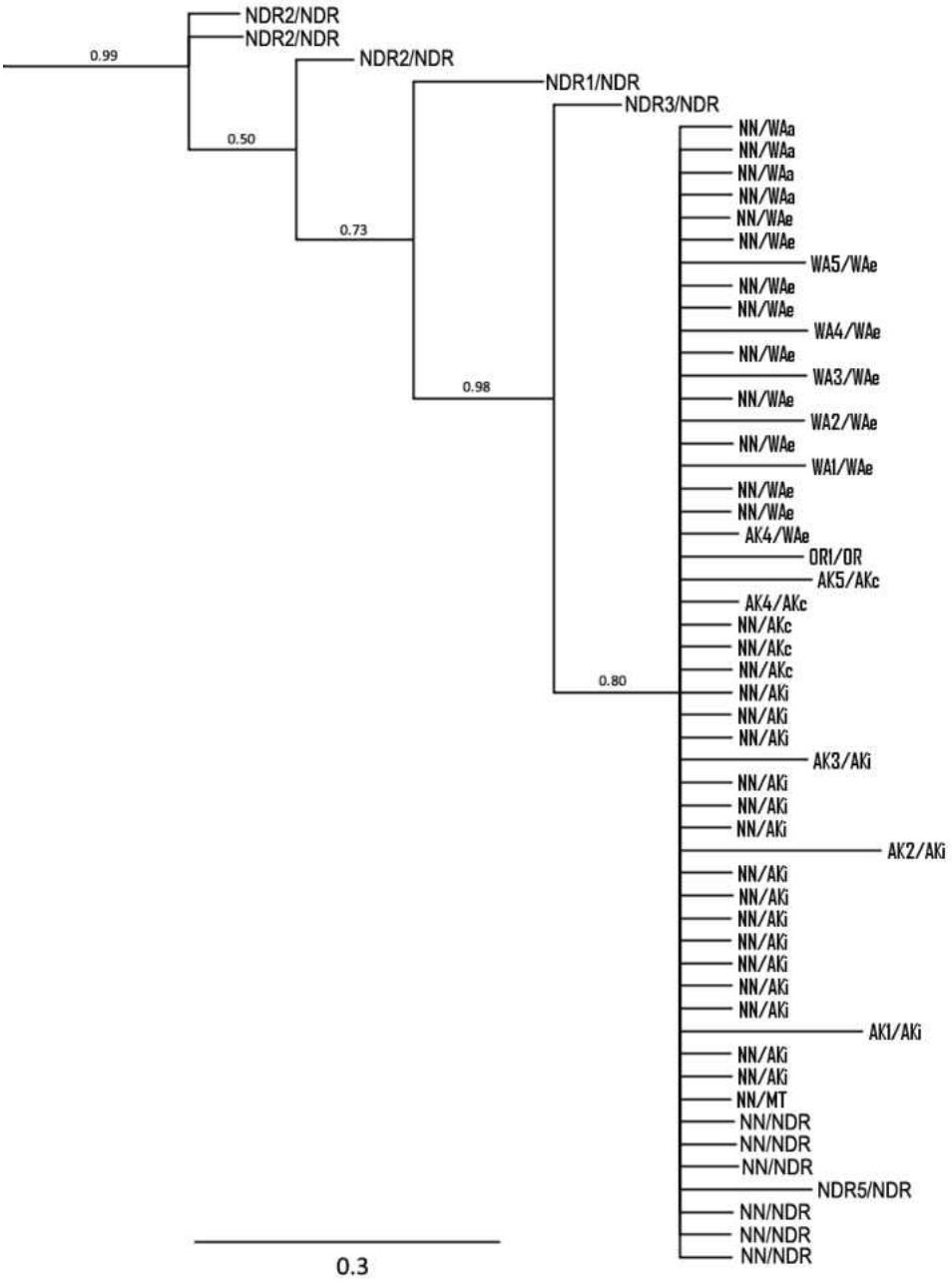

Figure 12. Phylogenetic tree of the Nearctic North clade using the without gap dataset and Bayesian Inference with 10,000,000 iterations. Only posterior probabilities of less than 1 are shown. Individual birds are listed with haplotype (NN,NDR1, NDR2, NDR3, NDR4, NDR5 definitions found in Table 8), then by location collected NDR - North Dakota Region; AKi – interior AK; AKc –Bering Sea coast, AK; WAo – vicinity of Olympia, WA; WAc – Pacific coast of WA; WAi – White island, WA; WAe – eastern WA; WAa – alpine tundra, Cascade mountains, WA.

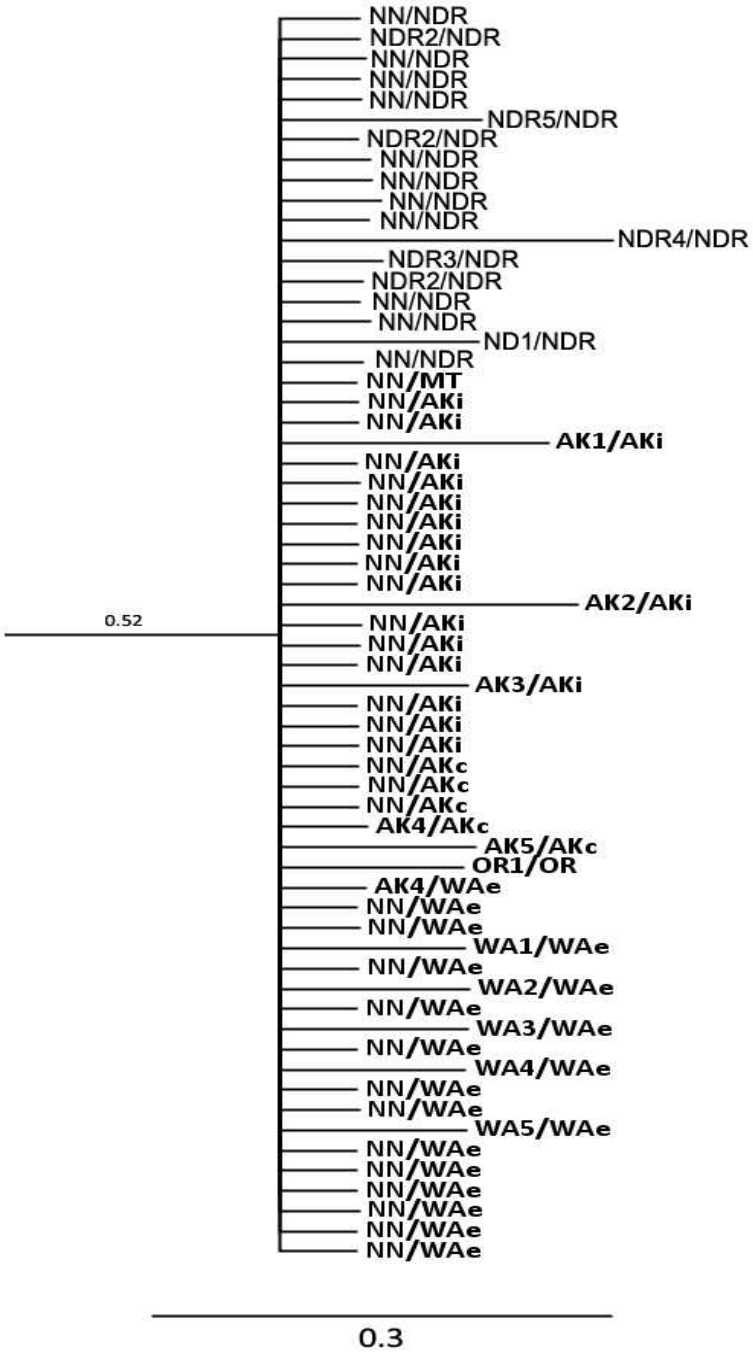

Figure 13. Phylogenetic tree of the Nearctic North clade using the gap dataset using Bayesian Inference with 10,000,000 iterations. Only posterior probabilities of less than 1 are shown. Individual birds are listed with haplotype (NN,NDR1, NDR2, NDR3, NDR4, NDR5 definitions found in Table 8), then by location collected NDR - North Dakota Region; AKi – interior AK; AKc –Bering Sea coast, AK; WAo – vicinity of Olympia, WA; WAc – Pacific coast of WA; WAi – White island, WA; WAe – eastern WA; WAa – alpine tundra, Cascade mountains, WA.

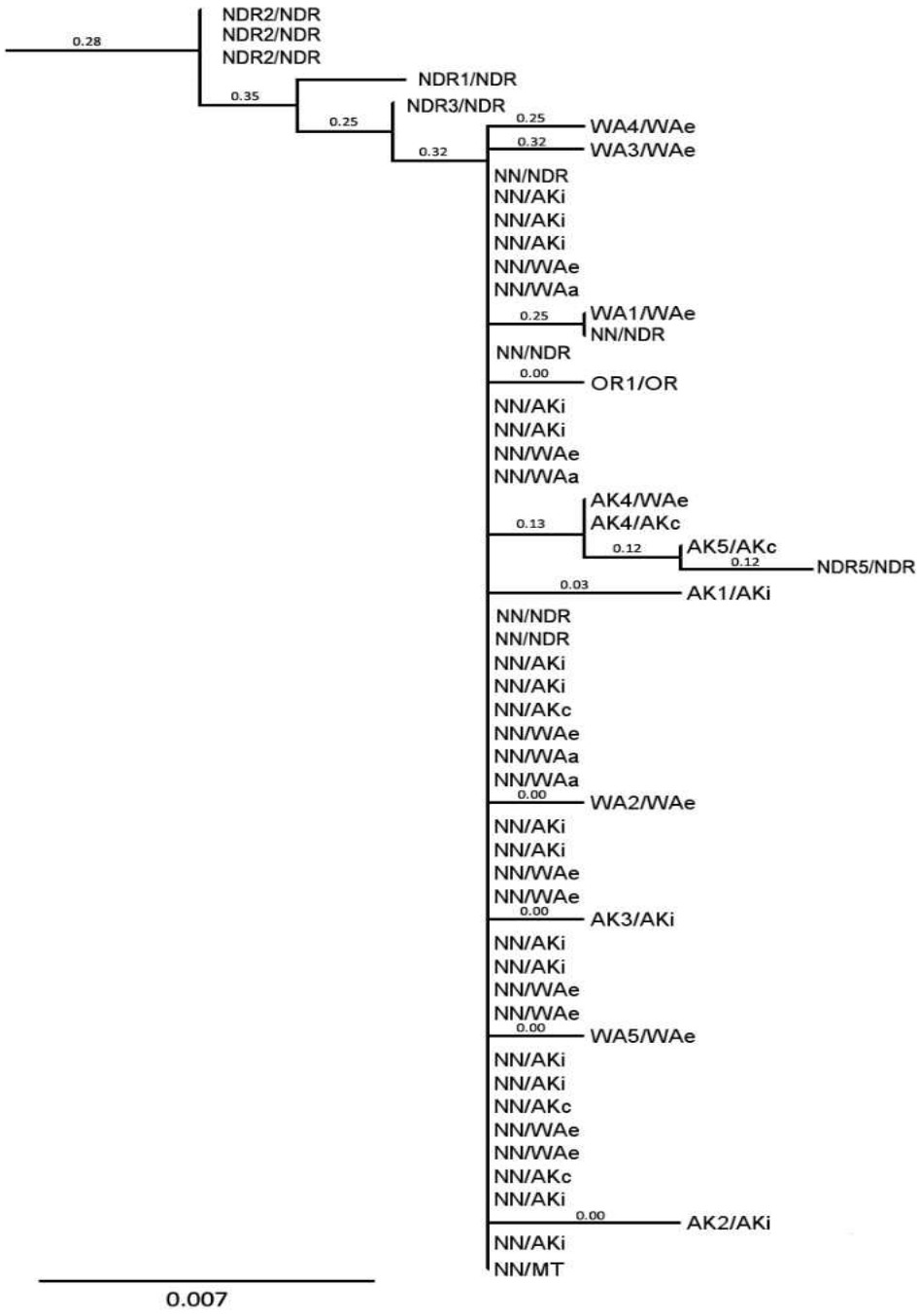

Figure 14. Phylogenetic tree of the Nearctic North clade using the without gap dataset using Maximum Likelihood with 10,000 bootstrap replications. Individual birds are listed with haplotype (NN,NDR1, NDR2, NDR3, NDR4, NDR5 definitions found in Table 8), then by location collected NDR - North Dakota Region; AKi – interior AK; AKc – Bering Sea coast, AK; WAo – vicinity of Olympia, WA; WAc – Pacific coast of WA; WAi – White island, WA; WAe – eastern WA; WAa – alpine tundra, Cascade mountains, WA.

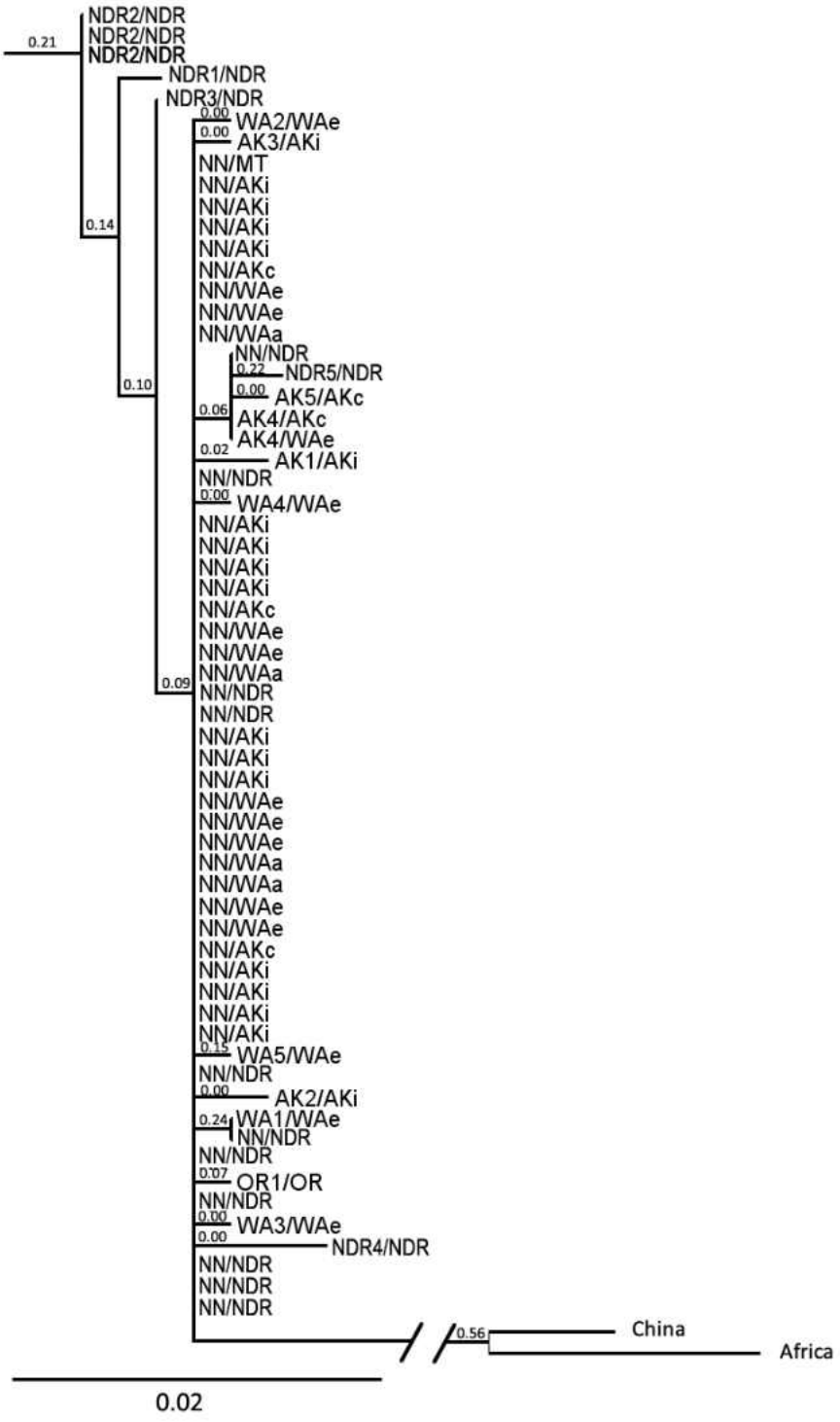

Figure 15. Phylogenetic tree of the Nearctic North clade using the Gap dataset using Maximum Likelihood with 10,000 bootstrap replications. Individual birds are listed with haplotype (NN,NDR1, NDR2, NDR3, NDR4, NDR5 definitions found in Table 8), then by location collected NDR - North Dakota Region; AKi – interior AK; AKc –Bering Sea coast, AK; WAo – vicinity of Olympia, WA; WAc – Pacific coast of WA; WAi – White island, WA; WAe – eastern WA; WAa – alpine tundra, Cascade mountains, WA.

Populations of comparison were based on sampling location in a previous publication by Drovetski et al. (2005) and geographic location of new sequences. The North Dakota regional population was found to have the highest nucleotide diversity (π_n = 0.0524 ± 0.0271) and the Oregon Population (OR) having the second highest level (π_n = 0.0073 ± 0.0051). All other populations of comparison had much lower amounts of genetic diversity (Table 9). This could be in part due to several populations represented by only a few individuals.

Population comparisons that had low F_{st} values were genetically similar and thus resulting in members of those populations being closer on the phylogenetic tree. Population comparisons that had high F_{st} values are genetically more different from each other and thus would be farther apart in phylogenetic reconstruction (Table 10).

Table 10. Pairwise Fst values (below diagonal) and their P-values (above diagonal), bold values are statically significant at a p-value of 0.05.

	NDR	MT	AKi	AKc	WAe	WAa	OR	CA	WAc	WAo	WAi	AZ
n	18	1	18	5	16	4	5	19	5	21	5	1
NDR	-	0.999	**0.014**	0.114	0.417	0.397	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.999
MT	-0.596	-	0.999	0.999	0.999	0.999	0.357	0.059	0.150	**0.035**	0.174	0.999
AKi	**0.152**	-1.000	-	0.066	0.096	0.999	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.999
AKc	0.122	-0.666	0.148	-	0.495	0.451	**0.035**	**0.000**	**0.012**	**0.000**	**0.006**	0.999
WAe	0.002	-1.000	0.005	-0.035	-	0.999	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.999
WAa	0.027	0.000	-0.136	0.111	-0.134	-	**0.047**	**0.000**	**0.006**	**0.000**	**0.011**	0.999
OR	**0.569**	0.344	**0.779**	**0.625**	**0.530**	**0.635**	-	**0.000**	**0.003**	**0.000**	**0.004**	0.999
CA	**0.739**	0.777	**0.855**	**0.812**	**0.722**	**0.819**	**0.550**	-	**0.000**	**0.000**	**0.000**	0.999
WAc	**0.878**	1.000	**0.966**	**0.963**	**0.831**	**1.000**	**0.824**	**0.810**	-	0.999	0.999	0.999
WAo	**0.928**	**1.000**	**0.980**	**0.987**	**0.902**	**1.000**	**0.935**	**0.883**	0.000	-	0.999	0.999
WAi	**0.878**	1.000	**0.966**	**0.963**	**0.831**	**1.000**	**0.824**	**0.810**	0.000	0.000	-	0.999
AZ	0.801	1.000	0.945	0.905	0.735	1.000	0.558	0.804	1.000	1.000	1.000	-

Population Location Definitions: NDR - North Dakota Region; AKi – interior AK; AKc –Bering Sea coast, AK; WAo – vicinity of Olympia, WA; WAc – Pacific coast of WA; WAi – White island, WA; WAe – eastern WA; WAa – alpine tundra, Cascade mountains, WA.

CHAPTER 5
DISCUSSION

Genetic diversity should be higher in the North Dakota Region than found in other regions of the United States. This is in part because of the overlapped range of three subspecies of the Horned Lark in the North Dakota Region (E.a. *alpestris*, E.a. *lecucoleama*, and E. a. *praticola*) (Dwight, 1890). To test this I used museum specimens collected over the past 120 years from the North Dakota region. By collecting sampling over a temporal range I hoped to limit any effect that year to year changes in the predominantly agricultural landscape or environmental stochasticity would have on specific subspecies abundance within the North Dakota region. This will provide a picture of genetic diversity though time rather than genetic diversity at discrete time points.

Five new haplotypes and three new SNPs were identified from museum specimens in this study. Horned Larks from the North Dakota region population had higher nucleotide diversity than other populations, and were all part of a single clade. These are the first DNA sequences of the Horned Lark from the state of North Dakota and the Prairie Pot Hole region. It is unlikely I sequenced any nuclear copies of mtDNA, because of the finding of established Horned Lark mtDNA ND2 SNPs, and no evidence of stop codons inside the sequences.

Museum specimens are an invaluable resource, preserving important morphological and genetic information across time. However, many museum specimens

may often be deemed unusable for historical population analysis because of preservation conditions. In my thesis I illustrate how conservatively using even small sections of a single gene, possibly the results of poor preservation, can give us glimpse of the historical genetic diversity of past populations. I base this conclusion on the results a comparative analysis of using of DNA sequences with uneven preservation in phylogenetic tree reconstruction in our Horned Lark study.

Molecular techniques provide researchers with the ability to amplify DNA from extremely small amounts of material, making it possible to limit damage to museum specimens during genetic sampling. Direct PCR allows researchers to avoid DNA extraction, which is a possible source of cross-transfer of DNA between samples and modern DNA contamination. No incubation period is needed with this method, and it cost far less than many DNA isolation kits.

The best statistical model to predict PCR success in a small collection at UND, constructed from a global GLM model, uses age as the best indicator of PCR success rate. This supports the conclusion that DNA in museum collections decays over time. As concluded by other researchers, age certainly plays a large role in PCR success from museum specimens (Töpfer et al. 2011a). However, age was not fully able to explain PCR success in my study and should not be used as the only factor examined when selecting specimens for a genetic study. The quality of initial preservation could have an influence on the longevity of material and thus the ability to amplify even short DNA fragments from it (Töpfer et al. 2011a and 2011b; Riesing et al. 2003, Roland et al. 2004, Spitzenberger et al. 2006). Other possible factors influencing PCR success could include the cause of death for a museum specimen, and time between death and preservation.

Locally collected specimens are frequently recovered as road kill. In many cases, information such as cause of death may be not cataloged for specimens (Graham et al. 2004, Wandeler et al. 2007). A more exhaustive sampling of avian museum specimens with complete records from death, including preservation and storage conditions would be needed to better develop a predictive model.

No statistical difference in the ability to amplify DNA from an individual species was observed, providing supporting evidence that sampling from local museums can be broadly applied to many bird species for historic genetic analysis. By using museum specimens from local collections for historic genetic analysis, the door opens for geographically and temporally based avian systemic and population studies covering specific geographic areas.

Sequence gaps in historical population level studies may be unavoidable due to uneven DNA preservation in museum specimens. This may be especially true when dealing with small local museum collections. The use of sequence information with gaps and the appropriate phylogenetic methods to deal with them is a highly debated subject (Lemmon et al. 2009; Simmons 2012; Wiens and Morrill 2011). The use of the database of previously sequenced Horned Lark ND2 genes should have limited the effect of issues associated with gap sequences in our phylogenetic analysis, because of the overwhelming 16:1 ratio of completed sequences to those with uneven preservation in my genetic study of the Horned Lark.

Rare haplotype frequency is likely overestimated because of the small sample size of the North Dakota region population. However, all available specimens from the region were used, limiting the possibility of finding more haplotypes from historic specimens in

this area. The limited number of specimens proved too small to accurately depict patterns of haplotype occurrence over space and time within the North Dakota region. Because of care and conditions in which many of the specimens for this study were kept, we were unable to accurately identify museum specimens to the subspecies level. However, global climate change could, or may have already impacted the future distribution of these subspecies even within North Dakota. Range shifts have already been documented in a number of wildlife species (Dawson et al. 2011). If subspecies within the Nearctic North clade has some adaptation for better surviving in the cold, they may one day lose habitat to southern Horned Lark subspecies expanding their range north under new climate conditions.

The last glacial maximum, theorized by Drovetski (2005) to have been the cause of the genetic isolation of the streaked Horned Lark would date the basal divergence of these clades earlier than the last ice age. Fossil evidence shows the species occurred in North America at least 23,000 years before present (Ducey, 1992). Nearctic clade divergence may have developed as a result of an adaptive radiation following Horned Lark emergence in North America. In my phylogenic tree we observe only four known surviving clades. Over time much of this radiation could have been eliminated, due to any number of stochastic environmental events, or simply remains unsampled. This radiation could be better explored by sampling from many more regions across North America and other continents.

CHAPTER 6
EPILOGUE

To understand genetic history of that species it is vital to reconstruct population fluctuations and current genetic diversity, the. A historic genetic survey is conducted by gathering samples from past populations found in museum collections. DNA obtained from historic specimens is valuable is because it represents genetic diversity before present day. These samples can be used as a benchmark to judge changing levels of genetic diversity in contemporary species and populations.

The non-invasive sampling method developed in this study, which can produce reliable mtDNA for other applications, should prove useful to those looking to use small avian museum specimens for genetic analysis. There will always be competing interests between those who seek to preserve museum specimens, and those who seek to use them for genetic studies. It is my hope that this method will provide some common ground in which no taxonomically important information is destroyed, meeting the goals of both groups.

Historic genetic monitoring of the Horned Lark in North Dakota has provided phylogenetic information about Horned Larks as a species with respect to clade distribution. These are the first DNA sequences from Horned Larks in this region of the country, and some of the oldest retrieved from small bird museum specimens. Using gap sequences within a single gene also had no effect on the overall clade groupings from our phylogenetic analysis. I found the genetic diversity of Horned Larks in this region is higher than found in other regions, possibly because of the overlapped range of three subspecies .This illuminates the possibility that there may be some genetic variation with

the region that might explain adaptation to Horned Larks in the NN clade to colder temperatures allowing them to out-compete possible immigrants despite a dynamic ecosystem landscape.

While conducting this study it became apparent the essential the need for quality control work in the laboratory to avoid errors. When DNA is amplified from only a few copies of DNA, the possibility from contamination from any source is a distinct possibility. The need for careful analysis of sequence information is critical for many any accurate interpretation of population or phylogenetic analysis and quantification of levels of genetic diversity. It is because these careful steps were taken that I am confident in my interpretation of the data.

Many new questions have been raised by this study. The geographically concordant clades of the Horned Lark open the door for a genomic level comparative study investigating possible environmental adaptation. The wide spread range yet localized groups of the Horned Lark make it an ideal candidate for such avian research. Much of the wide spread range of the Horned Lark still has no genetic information associated with it. Samples from other region have the potential to be quite informative in phylogenetic analysis. The possibility of including nuclear DNA or more mtDNA markers also opens the possibility for a better characterization of the phylogenetic distribution of this species. Finally, the current population of Horned Larks in the North Dakota region could be investigated to find out if historic mtDNA haplotypes are still present.

This study, while limiting in its scope, provides an important starting point for historic genetic monitoring. Many small local museum collections across the country can

be used to better define population genetic changes over time. This provides evidence for the continued need of museum specimens that are collected on the local scale. The use of museum specimen genetic studies allowing for quantification of genetic variation in relation to climate conditions will become more important in the coming years, as we face the onset of global environmental change. Assessment of this genetic response will prove vital in determining how to best manage wildlife populations to maintain genetic variability in the future.

APPENDIX A

Supplemental Table 1. Primers used in the sampling experiment.

Species	Locus	Amplicon Size (bp)	Forward Primer Sequence 5' to 3'	5'	3'	Reverse Primer Sequence 5' to 3'	5'	3'	Annealing Temperature Used (°C)
Franklin's Gull*	CR	330	TCCACCTGGTACGAAGC	33	50	GTCCAACAGTCATTAATAT	385	404	45
Horned Lark	ND2	181	GTGCAATGCTCATACTAACC	722	742	TGTTGCACAGTATGCGAGGC	903	883	45
Red-winged Blackbird	COI	224	CGGTGCATGAGCCGGAATAG	17	27	TTCGTCCCAATGCTATGTCA	241	221	45

* Primers from Krmpotich, unpublished

Supplemental Table 2. Primers used in Horned Lark genetic diversity study of the mtDNA ND2 gene.

Locus	Amplicon Size (bp)	Forward Primer Sequence 5' to 3'	5'	3'	Reverse Primer Sequence 5' to 3'	5'	3'	Annealing Temperature Used (°C)
ND2	138	ATGAACCCCCAAGCAAAACT	1	20	ATAGCTCGGGGGTGGTGGGA	139	119	55
ND2	190	TCTAGCCATCCTCCCTCTGA	110	130	TGAGGTTAGGATGAGACATG	300	280	53
ND2	179	ACACAAGCAACCGCCTCCGC	184	204	GGCCAGTGATGAGGGGGGAG	363	343	52
ND2	187	CTAGTCCCATTCCACTTCTG	321	341	GCCCTATTCAGCCCCCCAGA	508	488	53
ND2	147	TAACCGGCATGGCCATCCTA	460	480	ACAGGGTGAGTTTAGGGTTG	607	587	53
ND2	181	AAGTCCGAAAAATTCTGGCC	540	560	TTAGTGCAGGGGCTTTGGTT	721	701	53
ND2	181	GTGCAATGCTCATACTAACC	722	742	TGTTGCACAGTATGCGAGGC	903	883	45
ND2	138	ATTACACTACCCCCACACTC	904	924	TTAGATGGTGGGGGAGATTA	1042	1022	52

APPENDIX B

Supplemental Table 3. Full GLM results.

Model number	Intercept	Age	DNA isolation method	Bird Species	ng of DNA	260/280 ratio	k	Residual deviance	AICc	Delta	Model weight
2	2.073	-0.0262					2	33.54	37.99	0	0.275
18	1.389	-0.02642				0.568	3	32.93	39.85	1.866	0.108
12	-2.127	-0.034	+		0.013		4	30.42	40.02	2.037	0.099
10	1.908	-0.02703			0.001		3	33.26	40.18	2.193	0.092
4	2.073	-0.0262	+				3	33.54	40.47	2.479	0.08
6	4.055	-0.0362		+			4	31.17	40.77	2.784	0.068
28	-3.368	-0.03496	+		0.014	0.756	5	29.46	41.96	3.974	0.038
26	1.211	-0.02725			0.001	0.574	4	32.63	42.23	4.24	0.033
1	0.4055						1	40.38	42.52	4.537	0.028
20	1.382	-0.02642	+			0.568	4	32.93	42.53	4.543	0.028
16	-1.329	-0.04927	+	+	0.017		6	27.15	42.8	4.816	0.025
22	3.428	-0.03645		+		0.58	5	30.59	43.09	5.105	0.021
14	3.85	-0.037		+	0.001		5	30.89	43.39	5.398	0.018
8	4.055	-0.0362	+	+			5	31.17	43.67	5.684	0.016
17	-0.226					0.508	2	39.72	44.17	6.178	0.013
9	0.3178				5E-04		2	40.32	44.76	6.777	0.009
3	0.4055		+				2	40.38	44.83	6.838	0.009
32	-2.708	-0.05391	+	+	0.019	1.003	7	25.75	44.84	6.853	0.009
30	3.212	-0.03723		+	0.001	0.577	6	30.32	45.97	7.986	0.005
24	3.397	-0.03646	+	+		0.585	6	30.59	46.24	8.253	0.004
25	-0.3204				5E-04	0.509	3	39.65	46.58	8.591	0.004

Supplemental Table 4. Full GLM results continued.

Model number	Intercept	Age	DNA isolation method	Bird Species	ng of DNA	260/280 ratio	k	Residual deviance	AICc	Delta	Model weight
19	-0.2325		+			0.509	3	39.72	46.64	8.657	0.004
11	-1.52		+		0.005		3	39.72	46.64	8.657	0.004
5	1.099			+			3	39.81	46.74	8.75	0.003
27	-2.46		+		0.006	0.567	4	38.92	48.52	10.53	0.001
21	0.4485			+		0.533	4	39.13	48.73	10.74	0.001
13	1.008			+	5E-04		4	39.75	49.35	11.36	0.001
7	1.099		+	+			4	39.81	49.41	11.43	0.001
15	-1.048		+	+	0.006		5	39.01	51.51	13.52	0
29	0.3487			+	5E-04	0.53	5	39.07	51.57	13.58	0
23	0.4332		+	+		0.534	5	39.13	51.63	13.64	0
31	-1.91		+	+	0.007	0.588	6	38.18	53.84	15.85	0

APPENDIX C

This appendix contains the partial Horned Lark mtDNA sequences recovered from museum specimens in the North Dakota region. Sequences are listed in 70 base pair lines, and dash indicates that no information was recoverable for that specific nucleotide. Raw DNA sequences output chromatograms are also included for all sequenced sections.

Specimen UND-AM-1230
MtDNA ND2 Gene Sequence bases 1-1042

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----------------------------------------------------------------------

----------------------------------------TCTAGCCATCCTCCCTCTGATCTCAAAA

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCTCATCCTAACCTCA-----------------------------------------------

----------------------------------------------------------------------

----------------------------------------------------------------------

---------------------------------------------AAAAATTCTGGCCTTCTCCTCTATC

TCTCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCCTGTTAAACTTCTACCTA

TACACCCTAATAACTGCAGCCATCTTCCTCACCCTAAATTCAACCAACACCACAAAACTATCTACCCTA

ATAACTTCATGAACCAAAGCCCCTGCACT--------TGCTCATACTAACCCTACTATCCCTAGCAGGC

CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAAATAACT

CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

GCAAC-----------------------------------------------------------------

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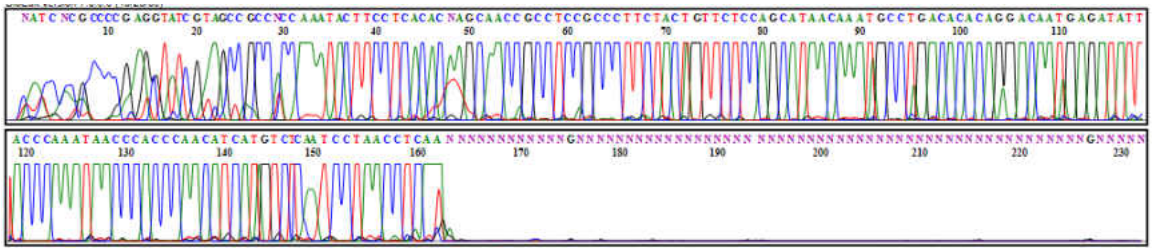
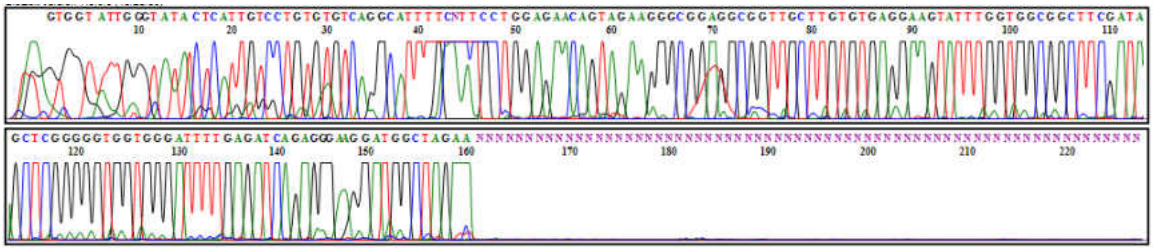
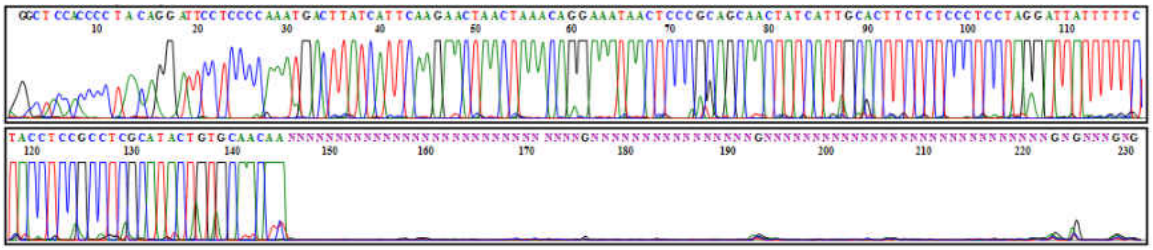
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



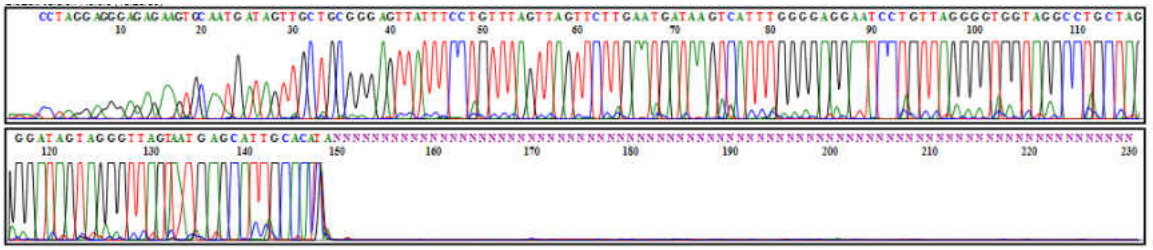
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen UND-AM-711
MtDNA ND2 Gene Sequence bases 1-1042

----------GCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCTCATCCTAACCTCAG----------

----------GTGCTAACCGGCATGGCCCTCCTATCC

GCAGCTCTGGGGGGCTGAATAGGGCTAAACCAAACACAAGTCCGAAAAATCCTGGCCTTCTCCTCTATC

TCTCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCCTGTTAAACTTCTACCTA

TACACCCTAATAACTGCAGCCATCTTCCTCACCCTAAATTCAACCAACACCACAAAACTATCTACCCTA

ATAACTTCATGAACCAAAGCCCCTGCACT--GTGCAATGCTCATACTAACCCTACTATCCCTAGCAGGC

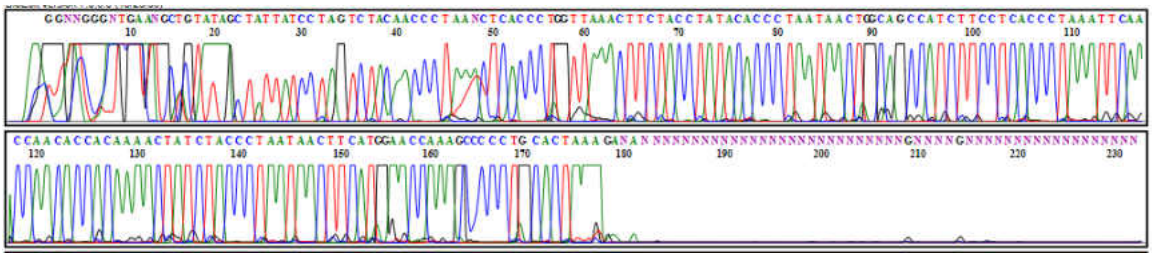
CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAGATAACT

CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

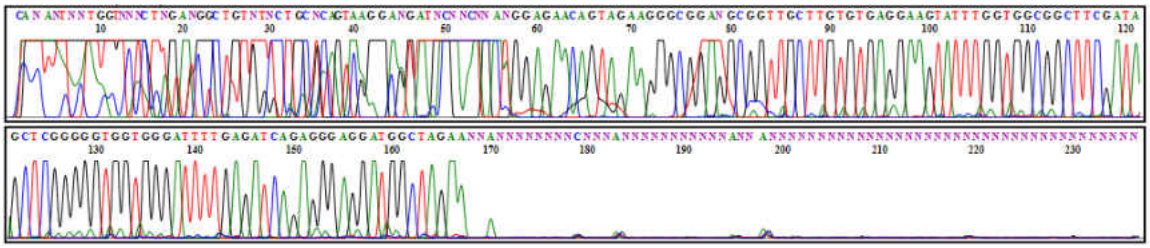
GCAACAATTACACTACCCCCACACTCCACAAATCACATAAAACAGTGGCATACCAACAAACCCATT---

--CTCTATTGCCATCCTAACC-TCCT----------

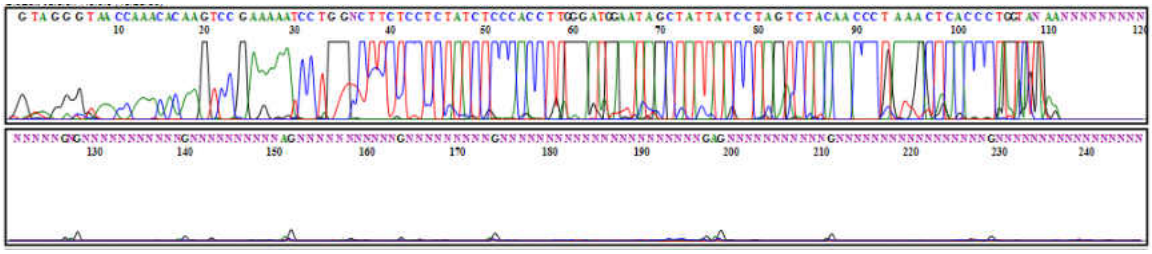
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



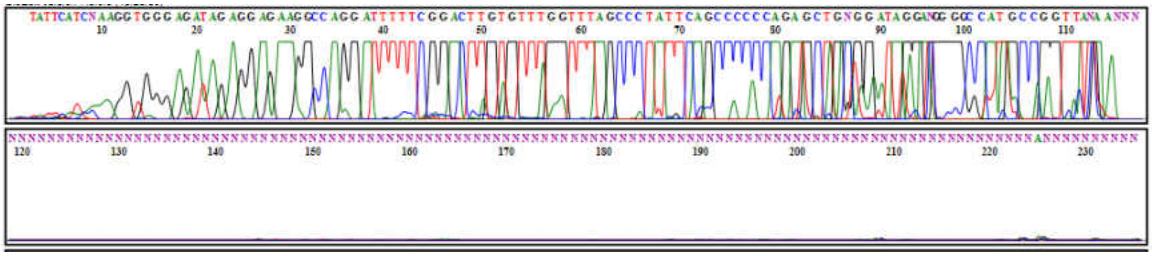
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



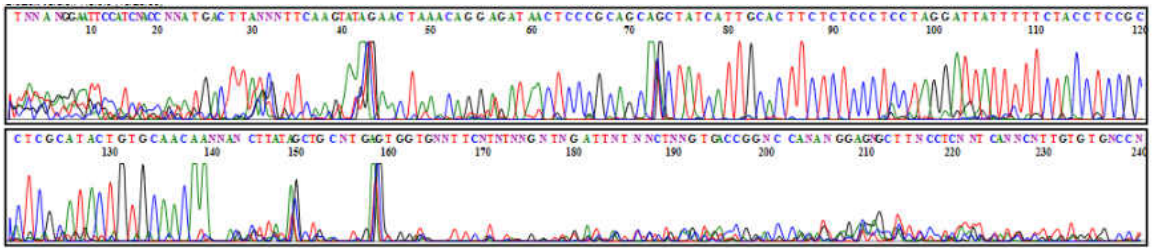
Chromatogram for Forward 460-607bp within the mtDNA ND2 gene.



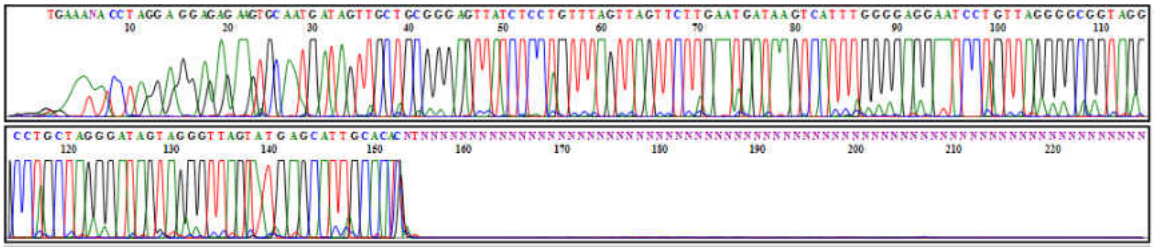
Chromatogram for Reverse 460-607bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen UND-AM-699
MtDNA ND2 Gene Sequence bases 1-1042

----------TCTAGCCATCCTCCCTCTGATCTCAAAA

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGCCCTT

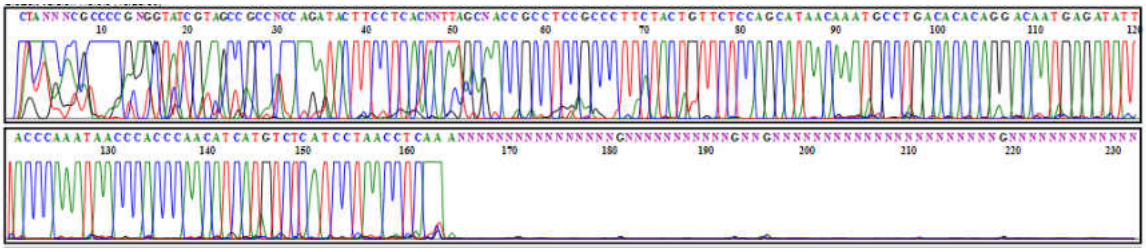
CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCTCA----------

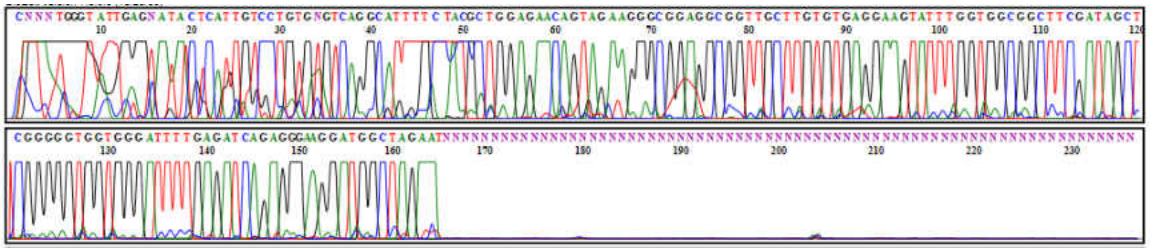
----------GTGCAATGCTCATACTAACCCTACTATCCCTAGCAGGC

CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTC----------

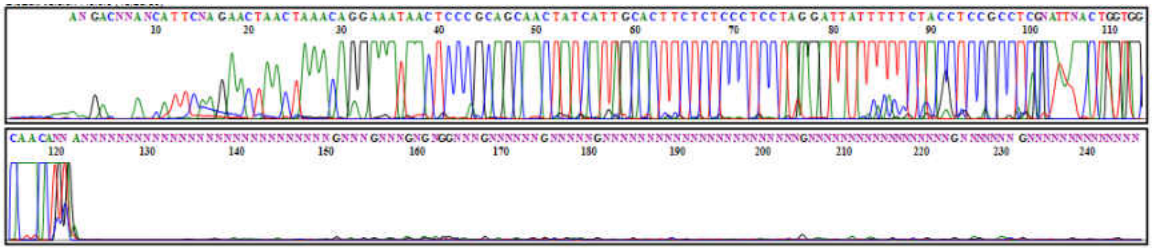
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



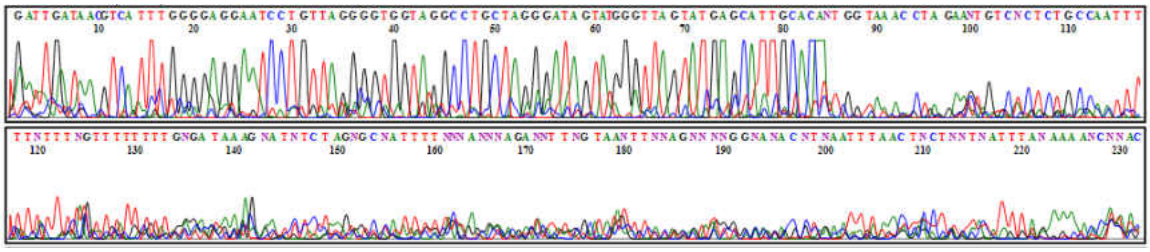
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen UND-AM-713
MtDNA ND2 Gene Sequence bases 1-1042

----------TCTAGCCATCCTCCCTCTGATCTCAAAA

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGAC----------

----------AGTGCTAACCGGC-TGGCCATCCTATCC

GCAGCTCTGGGGGGCTGAATAGGGCTAAACCAAACACAAGTCCGAAAAATTCTGGCCTTCTCCTCTATC

TCTCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCCTGTTAAACTTCTACCTA

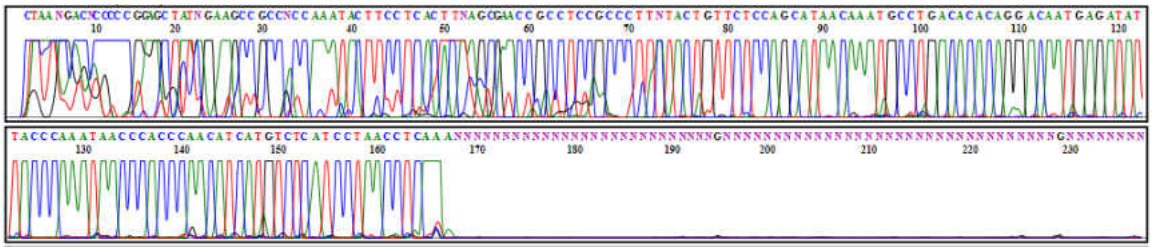
TACACCCTAATAACTGCAGCCATCTTCCTCACCCTAAATTCAACCAACACCACAAAACTATCTACCCTA

ATAACTTCATGAACCA----------GTGCAATGCTCATACTAACCCTACTATCCCTAGCAGGC

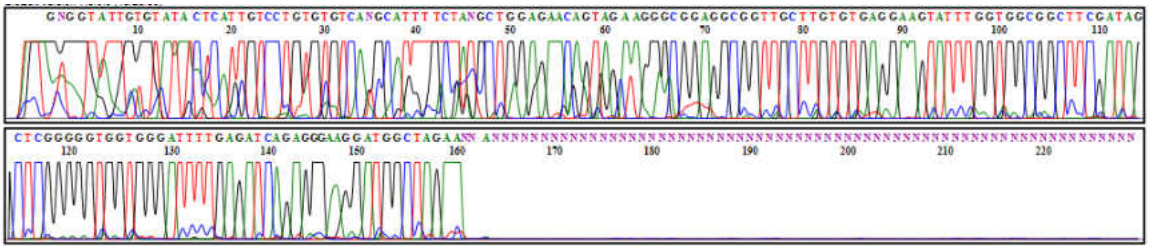
CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAAATAACT

CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAG----------

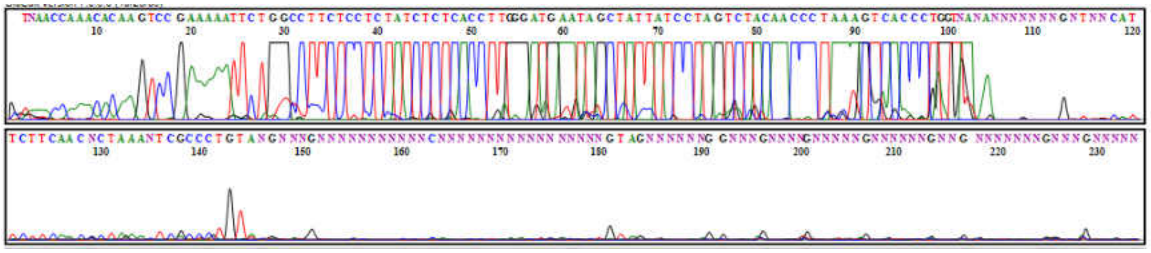
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



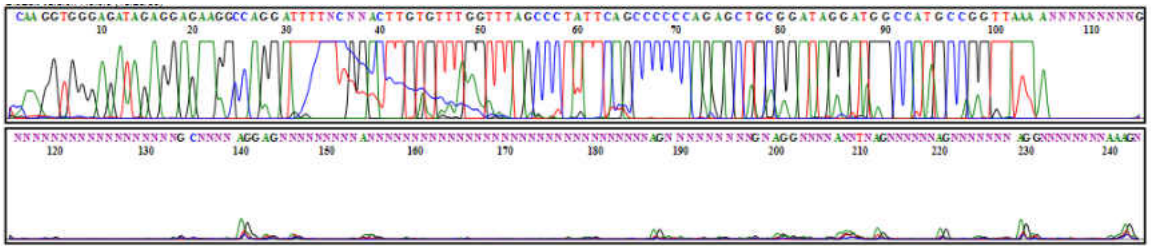
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



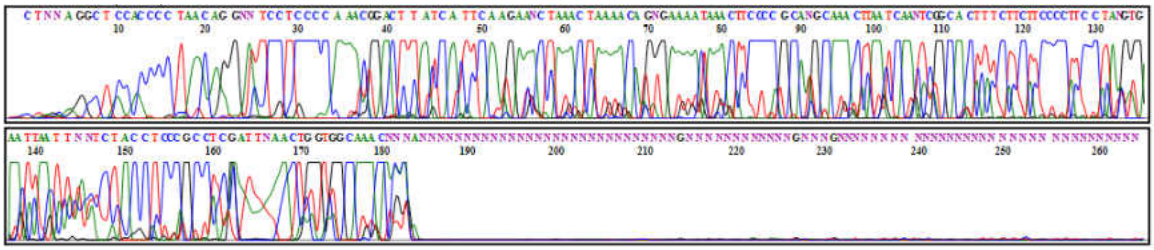
Chromatogram for Forward 460-607bp within the mtDNA ND2 gene.



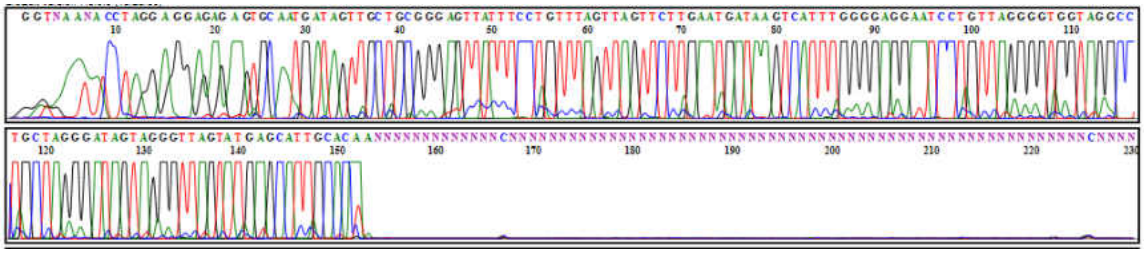
Chromatogram for Reverse 460-607bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen UND-AM-712
MtDNA ND2 Gene Sequence bases 1-1042

----------CCGCCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCTCATCCTAACCTCA----------

----------TAACCGGCATGGCCATCCTATCC

GCAGCTCTGGGGGGCTGAATAGGGCTAAACCAAACACAAGTCCGAAAAATCCTGGCCTTCTCCTCTATC

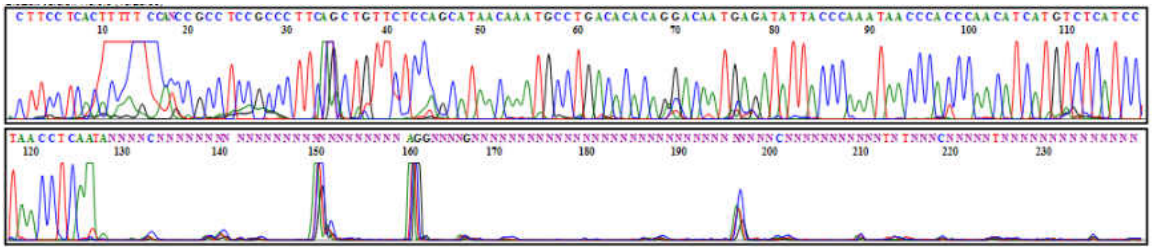
----------TGCAATGCTCATACTAACCCTACTATCCCTAGCAGGC

CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAGATAACT

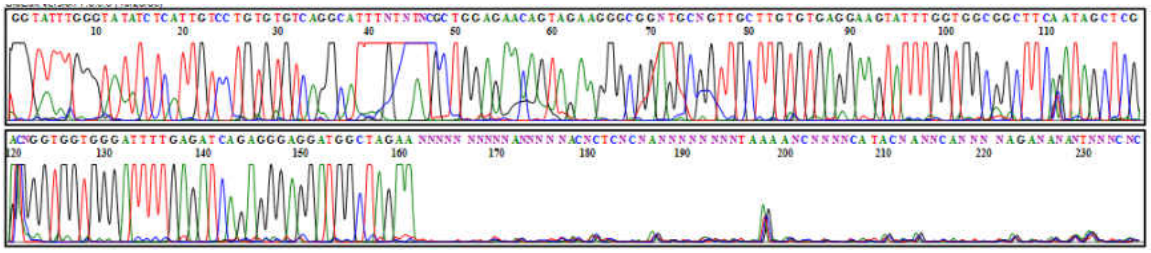
CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

GCAACA----------

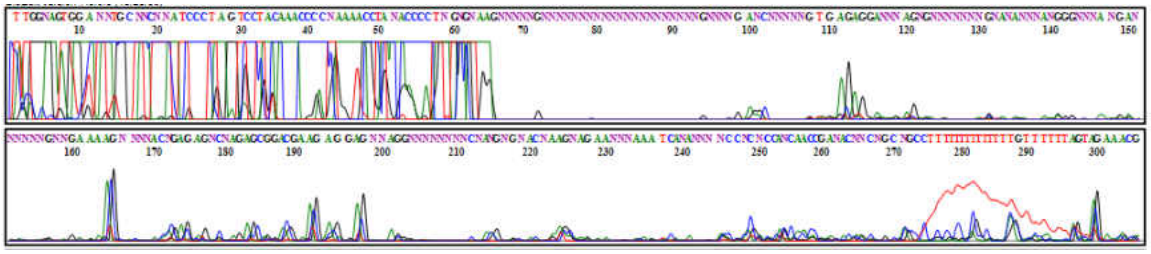
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



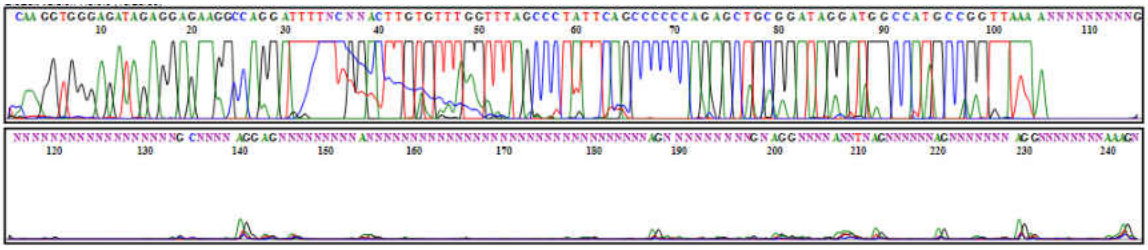
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



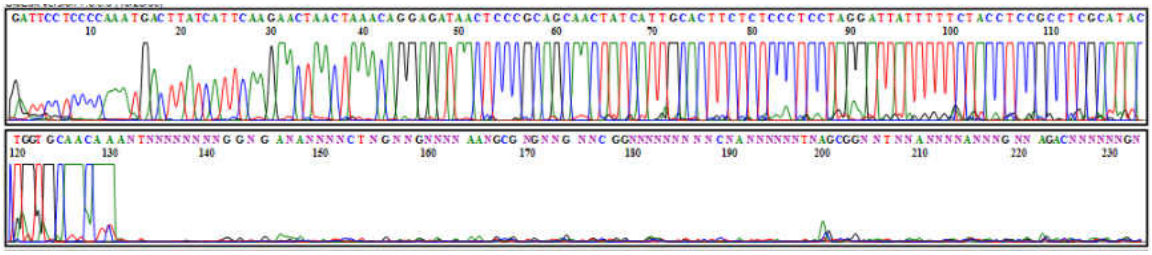
Chromatogram for Forward 460-607bp within the mtDNA ND2 gene.



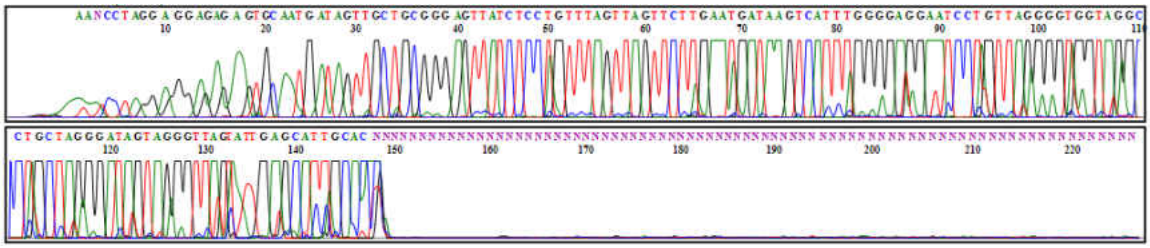
Chromatogram for Reverse 460-607bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen UND-AM-714
MtDNA ND2 Gene Sequence bases 1-1042

----------TCTGATCTCAAAA

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCTCATCCTAACCTCA----------

----------CGAAAAATTCTGGCCTTCTCCTCTATC

TCTCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCCTGTTAAACTTCTACCTA

TACACCCTAATAAC----------

----------GTGCAATGCTCATACTAACCCTACTATCCCTAGCAGGC

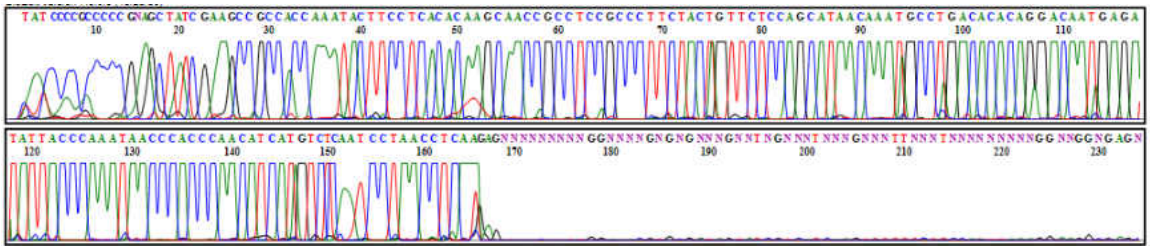
CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAGATAACT

CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

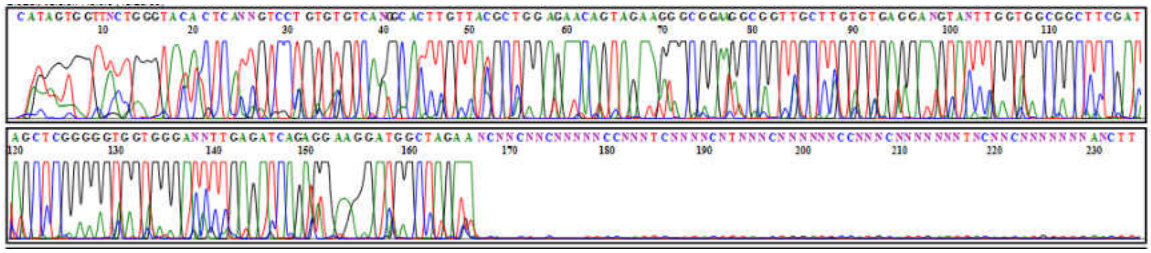
GCAACA---ACACTACCCCCACACTCCACAAATCACATAAAACAGTGGCATACCAACAAACCCATTCC-

---

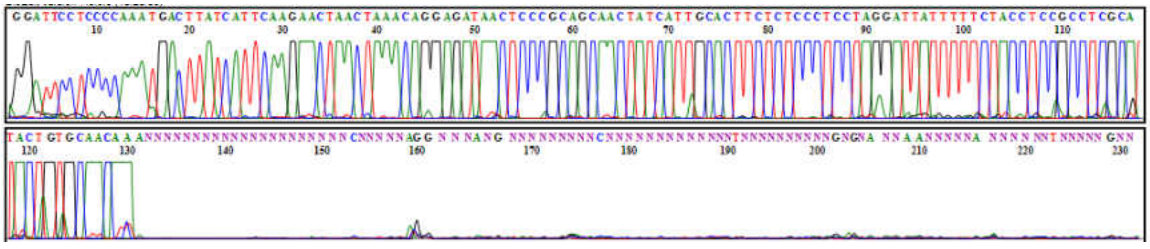
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



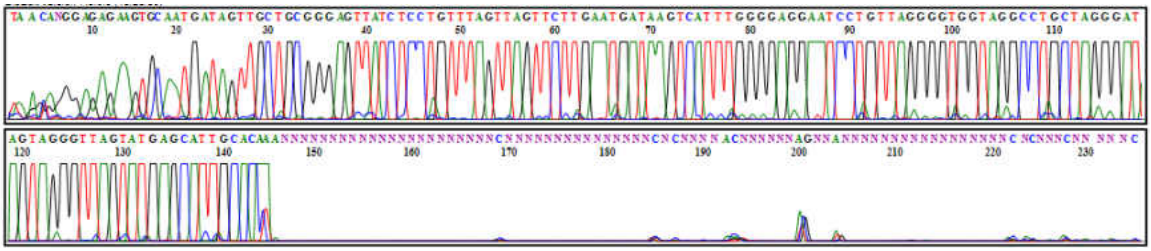
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen UND-AM-698
MtDNA ND2 Gene Sequence bases 1-1042

----------TCTAGCCATCCTCCCTCTGATCTCAAAA

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGTCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACATACAGGACAATGAGATATTACCCAAATAACCCACCCA

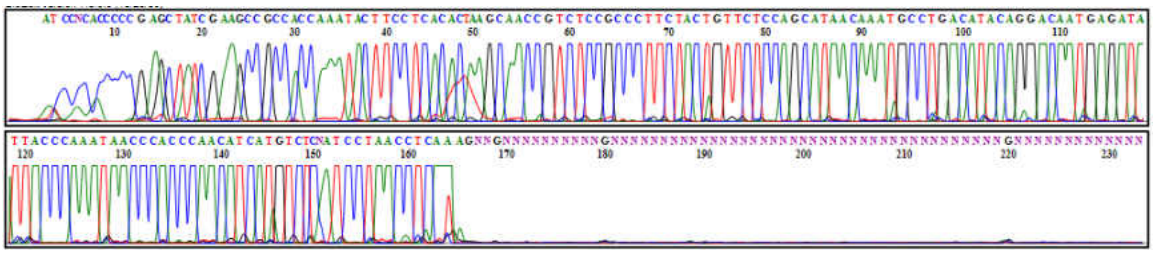
ACATCATGTCTCATCCTAACCTCA----------

----------GTGCAATGCTCATACTAACCCTACTATCCCTAGCAGGC

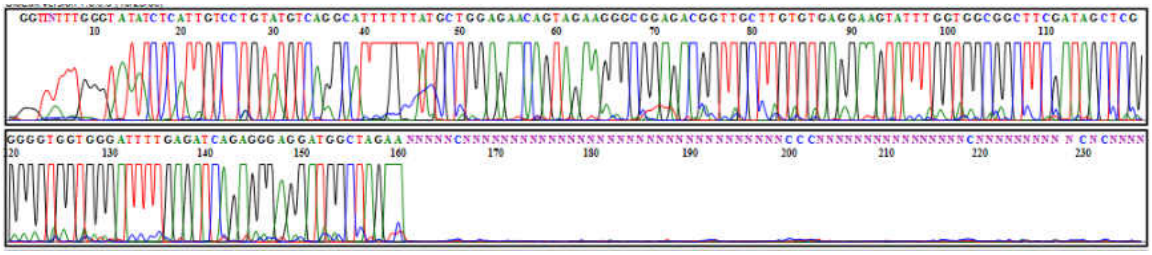
CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAAATAACT

CCCGCAGCAACTATCATTGCACT----------

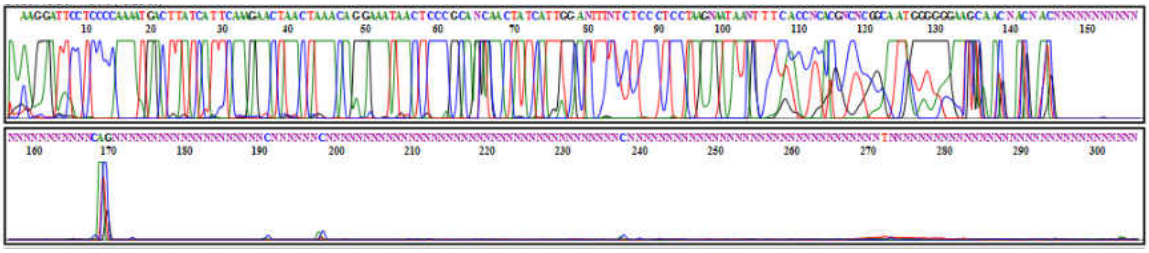
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



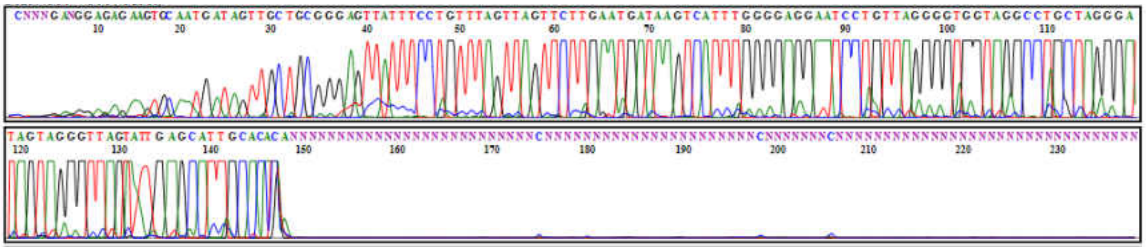
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen UND-AM-701
MtDNA ND2 Gene Sequence bases 1-1042

----------ATACTTCCTCACACAAGCAACCGCCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCTCATCCTAACCTC----------

----------AAGTCCGAAAAATTCTGGCCTTCTCCTCTATC

TCTCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCCTGTTAAACT-CTACCTA

TACACCCTAATAACTGCAGCCATCTTCCTCACCCTAAACTCAACCAACACCACAAAACTATCTACCCTA

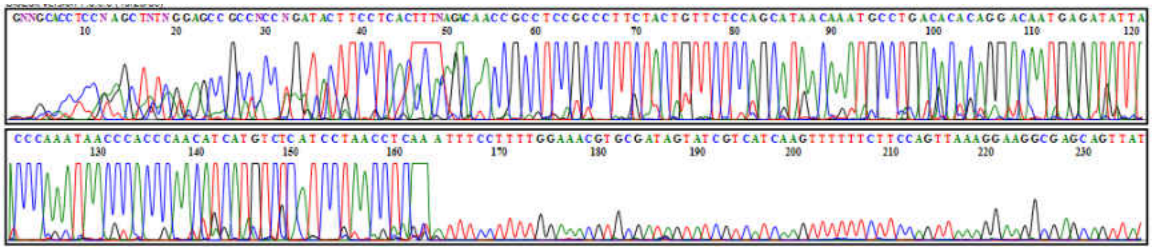
ATAACTTCATGAACCAAAGCCCCTGCACTAA----------

----------ACTAACTAAACAGGAAATAACT

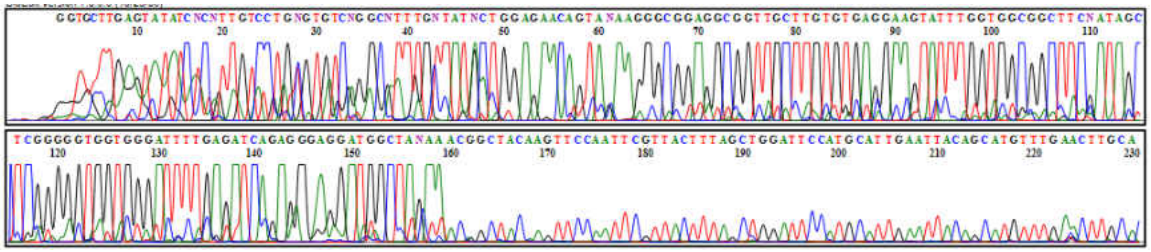
CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTG-

G----------

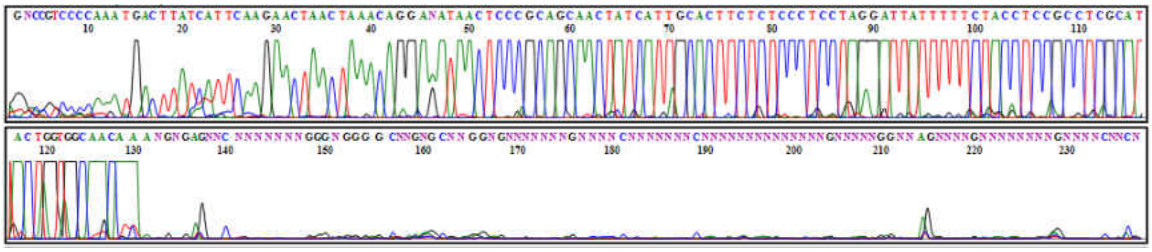
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



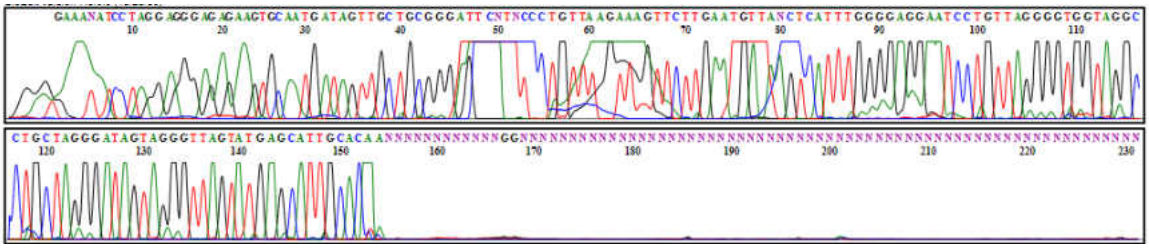
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.

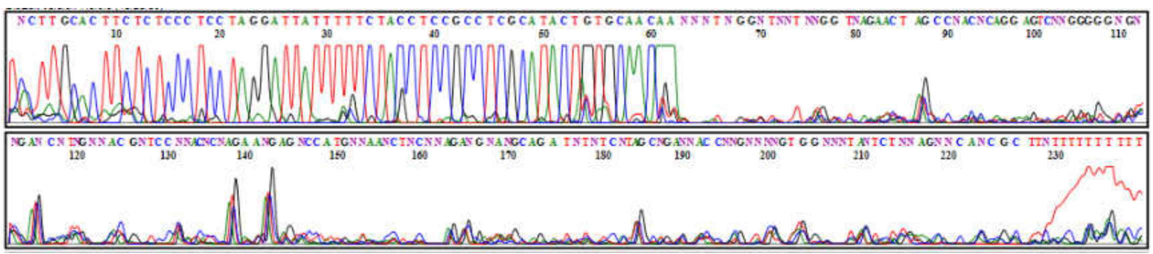


Specimen UND-AM-704
MtDNA ND2 Gene Sequence bases 1-1042

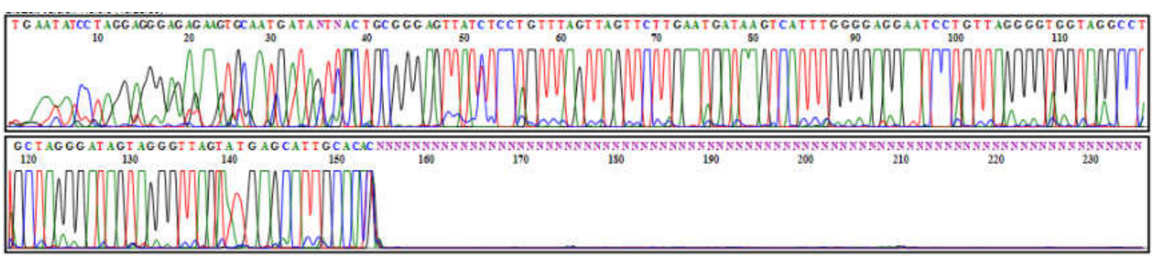
---GCA------ATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

GCAACA--

Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen UND-AM-709
MtDNA ND2 Gene Sequence bases 1-1042

---TATCC

GCAGCTCTGGGGGGCTGAATAGGGCTAAACCAAACACAAGTCCGAAAAATTCTGGCCTTCTCCTCTATC

TCTCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAA-------------------------

----------CCAACACCACAAAACTATCTACCCTA

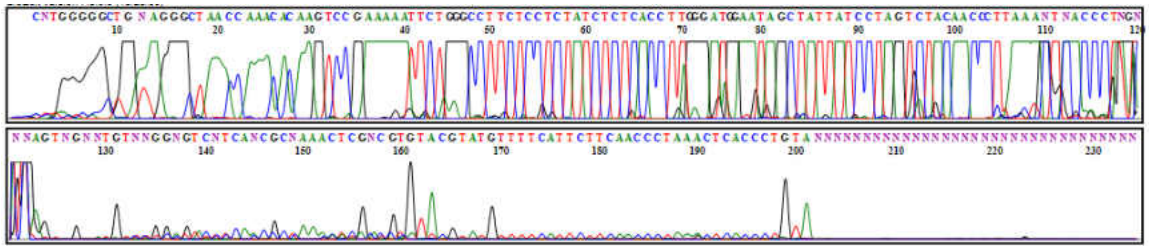
ATAACTTCATGAACCAAAGCCCCTGCACTA----------

----------AGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAAATAACT

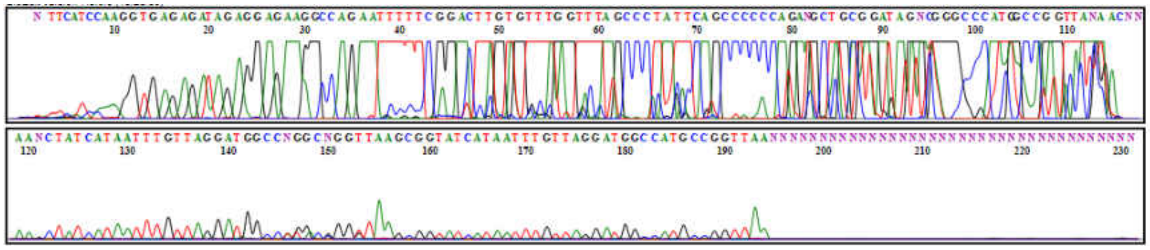
CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

GCAACA----------

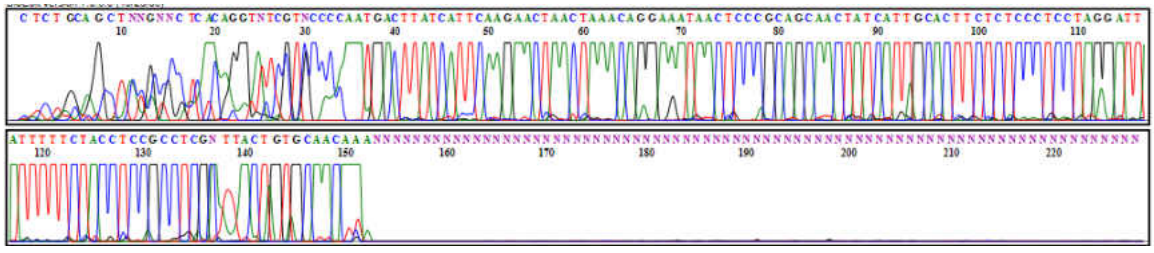
Chromatogram for Forward 460-607bp within the mtDNA ND2 gene.



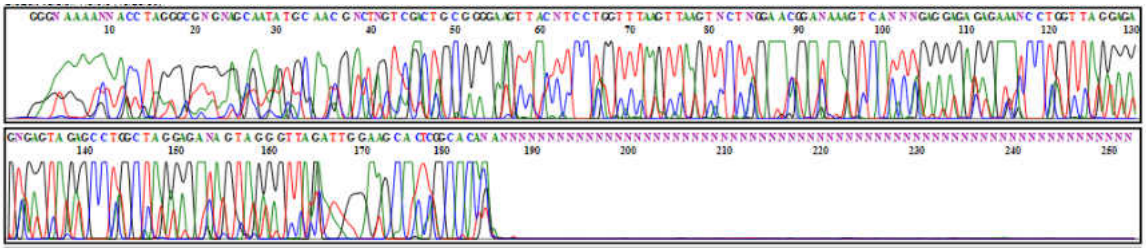
Chromatogram for Reverse 460-607bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen UND-AM-702
MtDNA ND2 Gene Sequence bases 1-1042

----------TCTAGCCATCCTCCCTCTGATCTCAAAA

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGC----

----

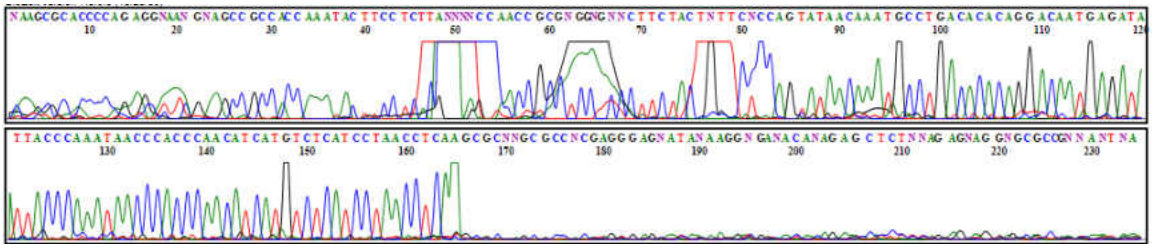
----------TAACCCTACTATCCCTAGCAGGC

CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAAATAACT

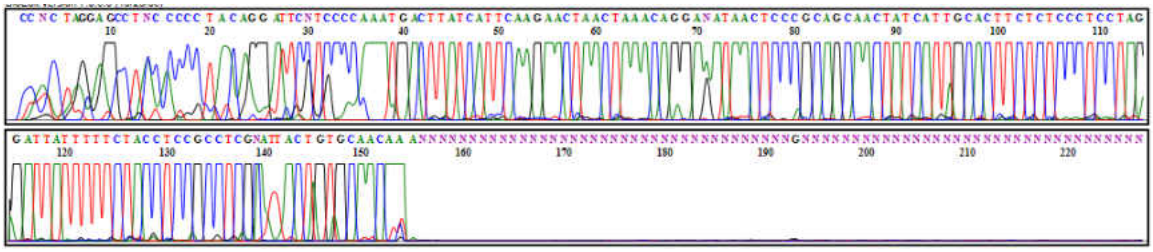
CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

GCAACA----------

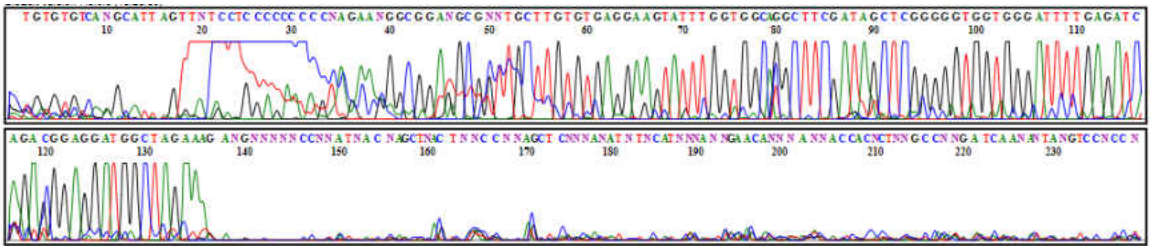
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



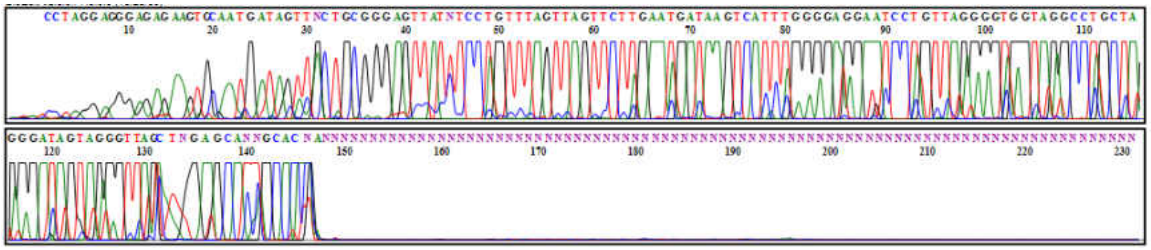
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen UMC-493
MtDNA ND2 Gene Sequence bases 1-1042

----------CTAGCCATCCTCCCTCTGATCTCAAAA

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCT--TCCTAACCTCA----------

----------AACCGGCATGGCCATCCTATCC

GCAGCTCTGGGGGGCTGAATAGGGCTAAACCAAACACAAGTCCGAAAAATCCTGGCCTTCTCCTCTATC

TCCCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCCTGTTAAACTTCTACCTA

TACACCCTAATAACTGCAGCCATCTTCCTCACCCTAAACTCAACCAACACCACAAAACTATCTACCCTA

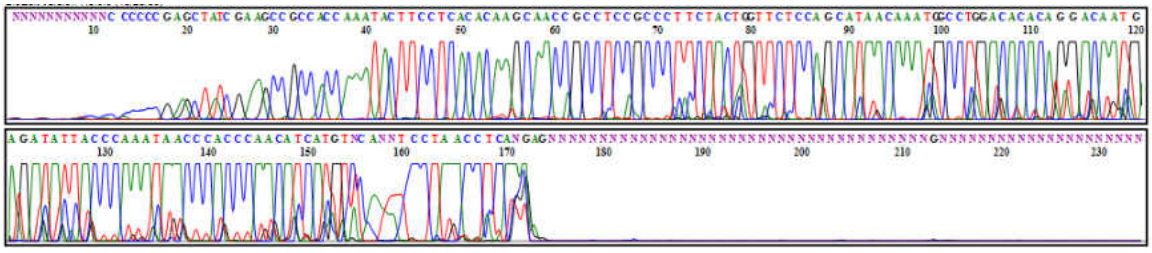
ATAACTTCATGAACCAAAGCCCCTGCACTAA-TGCAATGCTCATACTAACCCTACTATCCCTAGCAGGC

CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAGATAACT

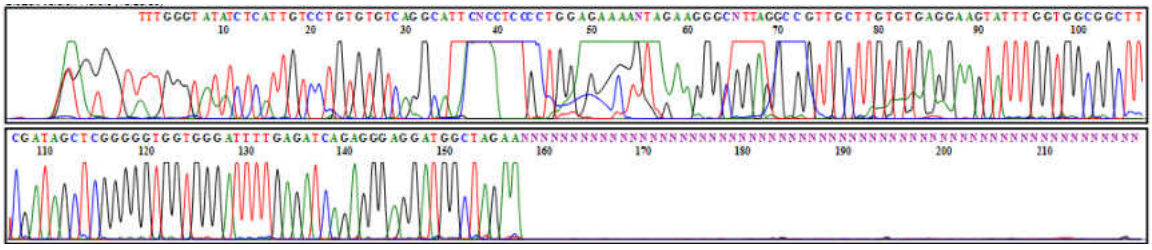
CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

GCAACAA----------

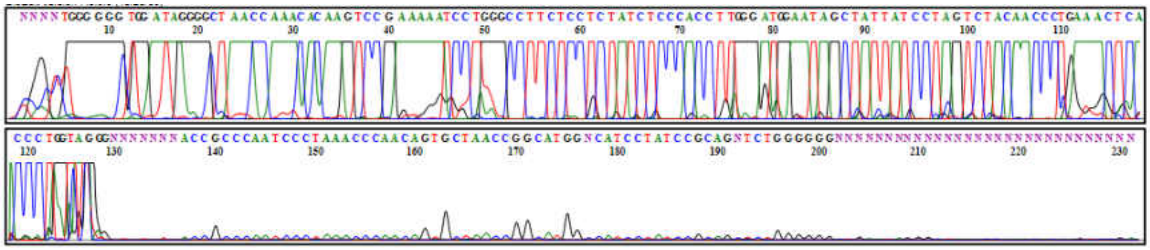
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



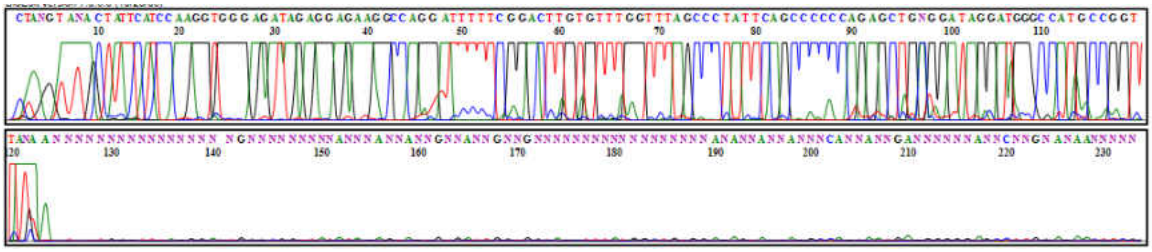
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



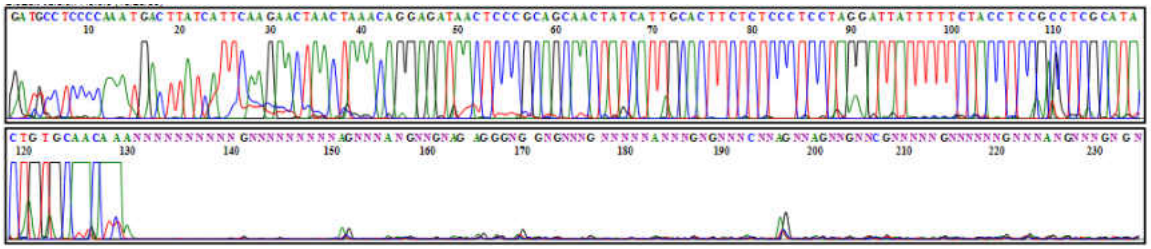
Chromatogram for Forward 460-607bp within the mtDNA ND2 gene.



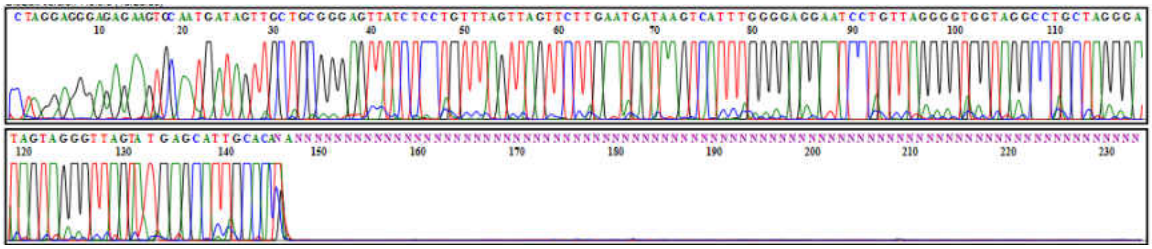
Chromatogram for Reverse 460-607bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen UMC-492
MtDNA ND2 Gene Sequence bases 1-1042

----------TCTAGCCATCCTGCCTCTGATCTCAAAA

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGCCCT-

---

----------TAACCGGCATGGCCATCCTATCC

GCAGCTCTGGGGGGCTGAATAGGGCTAAACCAAACACAAGTCCGAAAAATTCTGGCCTTCTCCTCTATC

TCTCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCCTGT----------

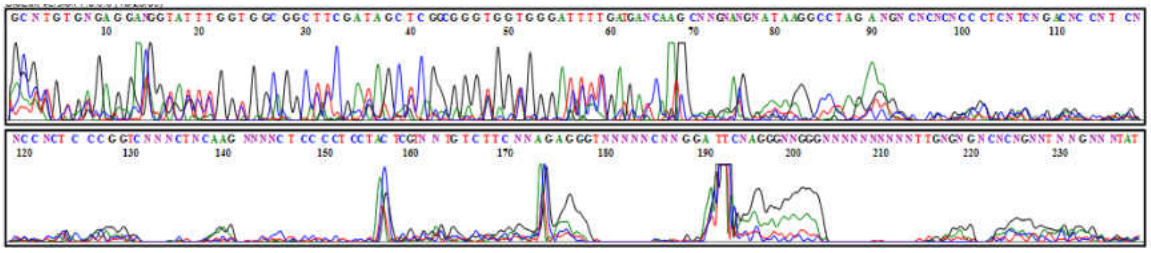
---

----CACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAAATAACT

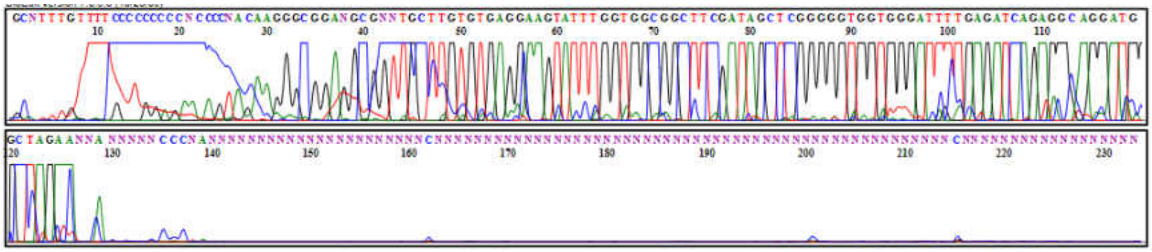
CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

GCAACAA---

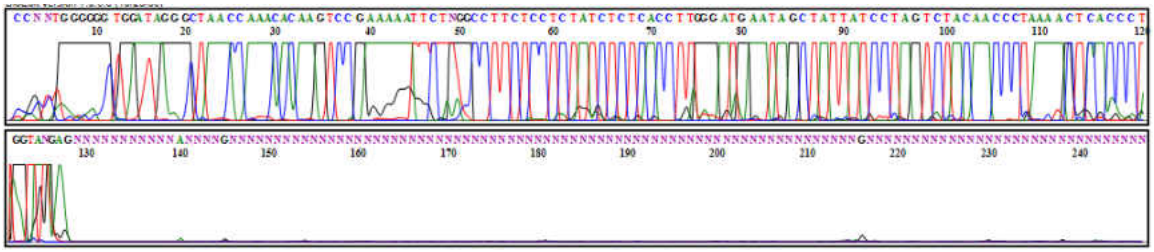
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



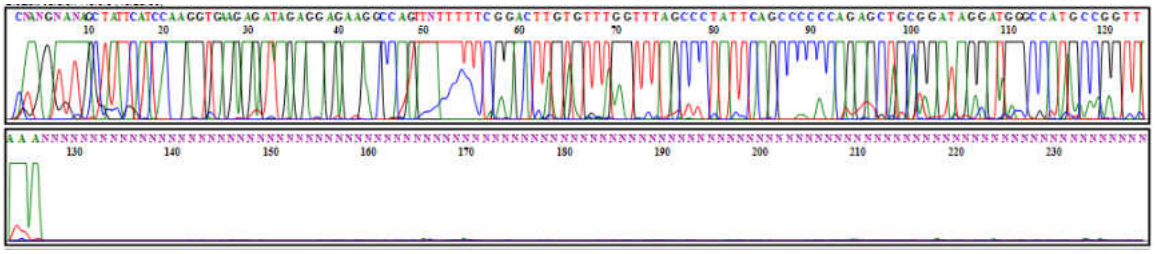
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



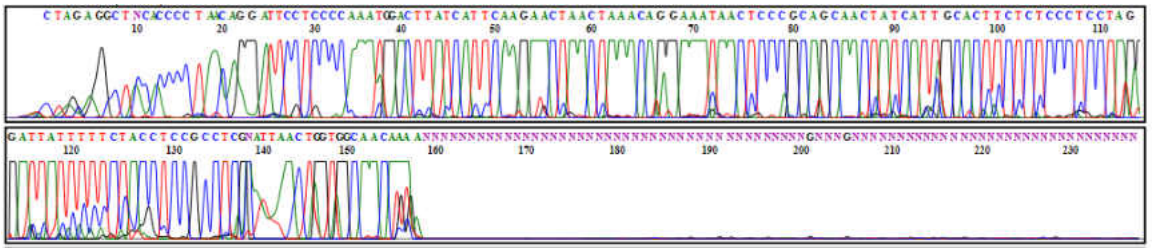
Chromatogram for Forward 460-607bp within the mtDNA ND2 gene.



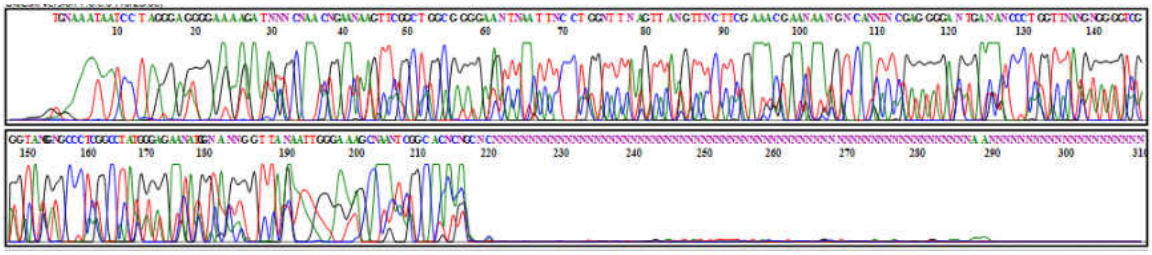
Chromatogram for Reverse 460-607bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen NDSU-2298
MtDNA ND2 Gene Sequence bases 1-1042

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CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCTCATCCTAACCTCA----------

----------TAACCGGCATGGCCATCCTATCC

GCAGCTCTGGGGGGCTGAATAGGGCTAAACCAAACACAAGTCCGAAAAATTCTGGCCTTCTCCTCTATC

TCTCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCC----------

---

----------GTGCAATGCTCATACTAACCCTACTATCCCTAGCAGGC

CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAAATAACT

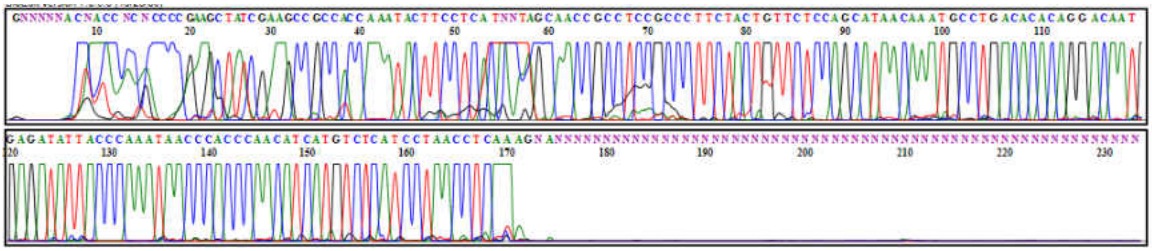
CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

GCAACAATTACACTACCCCCACACTCCACAAATCACATAAAACAGTGGCATACCAACAAACCCATTCCC

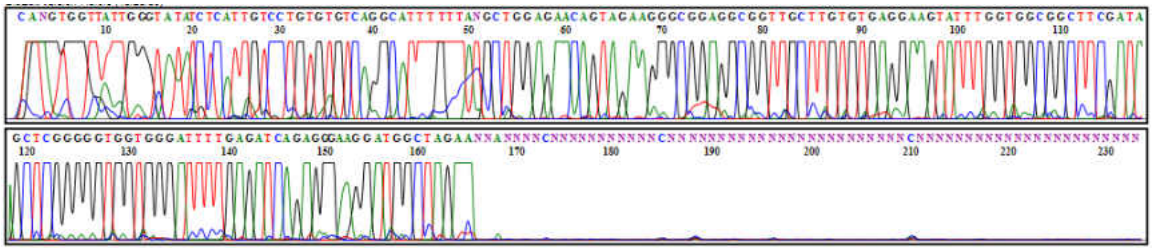
TCCTCTATTGC-ATCCTAACCGTCCTATCCACGACACTCCTCCCTCTATCCCCCATAATCTCCCCCACC

ATCTAA

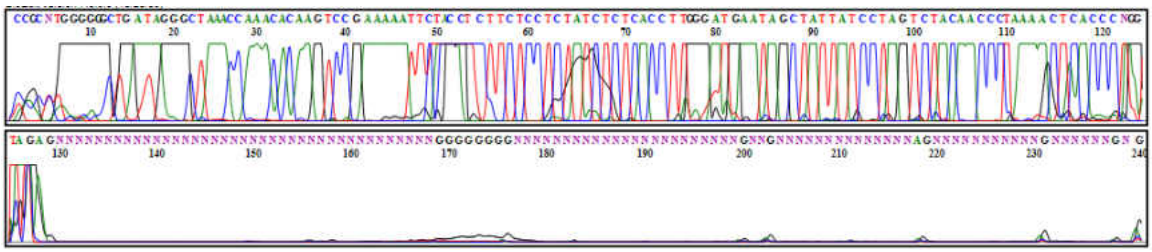
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



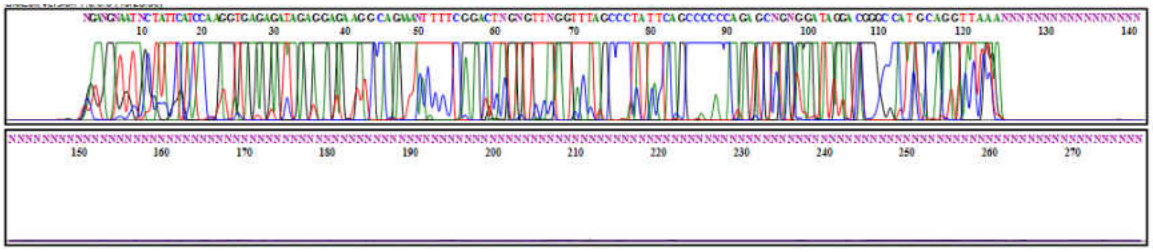
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



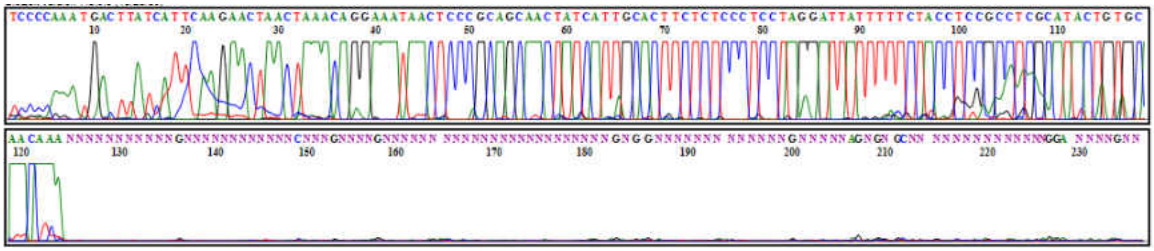
Chromatogram for Forward 460-607bp within the mtDNA ND2 gene.



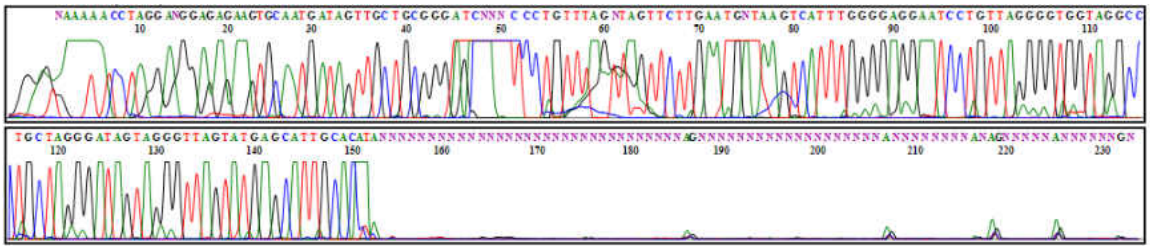
Chromatogram for Reverse 460-607bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen NDSU-2255
MtDNA ND2 Gene Sequence bases 1-1042

----------CTAGCCATCCTCCCTCTGATCTCAAAA

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCT-ATCCTAACCTCA----------

----------TAACCGGCATGGCCATCCTATCC

GCAGCTCTGGGGGGCTGAATAGGGCTAAACCAAACACAAGTCCGAAAAATTCTGGCCTTCTCCTCTATC

TCTCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCCTGTTAAACTTCTACCTA

TACACCCTAATAACTGCAGCCATCTTCCTCACCCTAAATTCAACCAACACCACAAAACTATCTACCCTA

ATAACTTCATGAACCAAAGCCCCTGCACTA-GTGCAATGCTCATACTAACCCTACTATCCCTAGCAGGC

CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAAATAACT

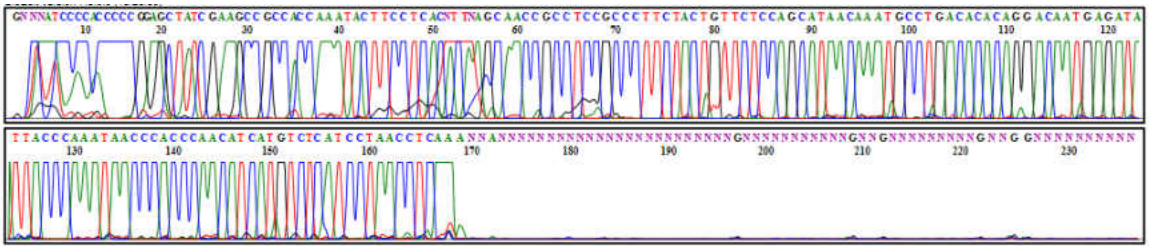
CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

GCAAC----------ACCAACAAACCCATTCCC

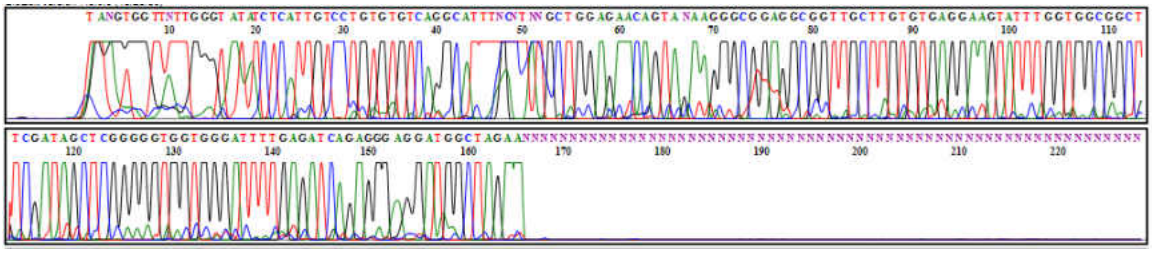
TCCTCTATTGCCATC--AACCGTCCTATCCACGACACTCCTCCCTCTATCCCCCATAATCTCCCCCACC

ATCTAA

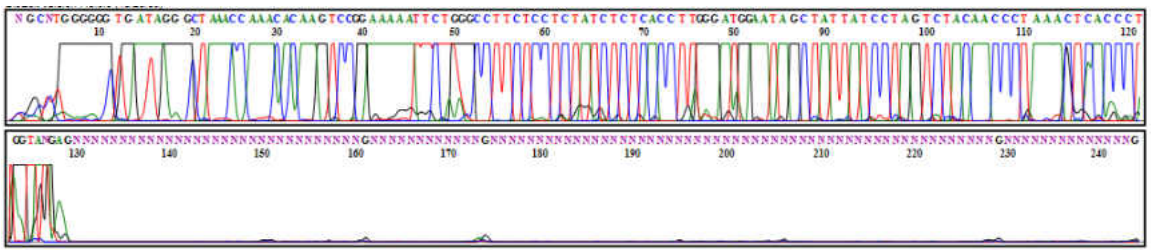
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



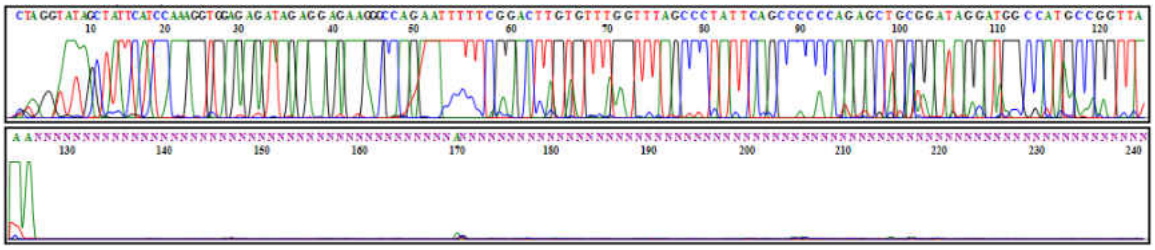
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



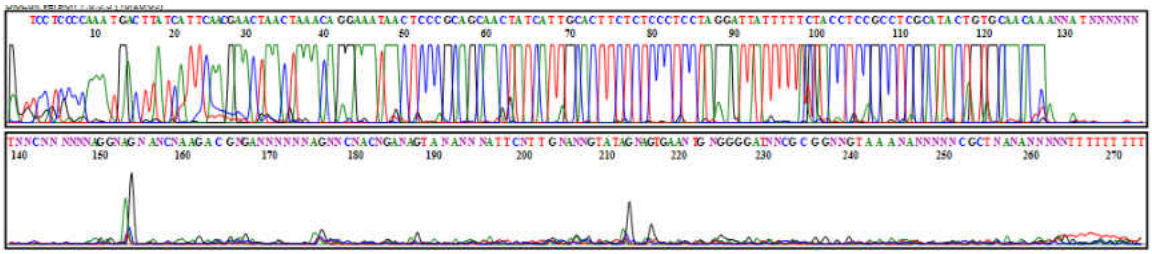
Chromatogram for Forward 460-607bp within the mtDNA ND2 gene.



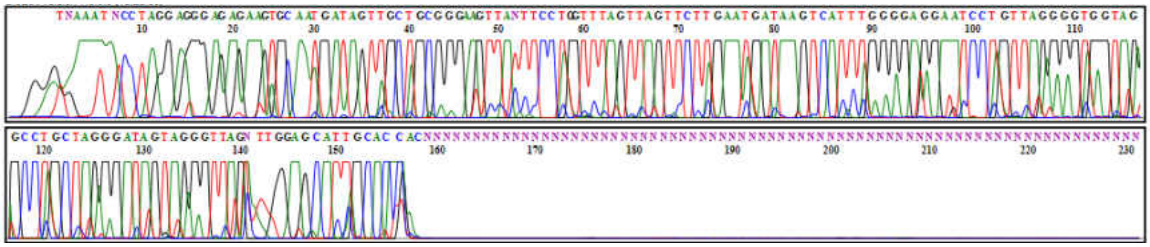
Chromatogram for Reverse 460-607bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen NDSU-496
MtDNA ND2 Gene Sequence bases 1-1042

----------CTAGCCATCCTCCCTCTGATCTCAAAA

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCTCATCCTAACCTCA----------

----------TAACCGGCATGGCCATCCTATCC

GCAGCTCTGGGGGGCTGAATAGGGCTAAACCAAACACAAGTCCGAAAAATTCTGGCCTTCTCCTCTATC

TCTCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCCTGT----------

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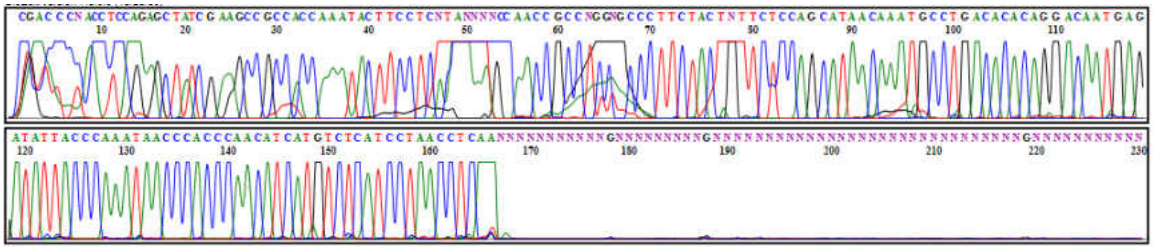
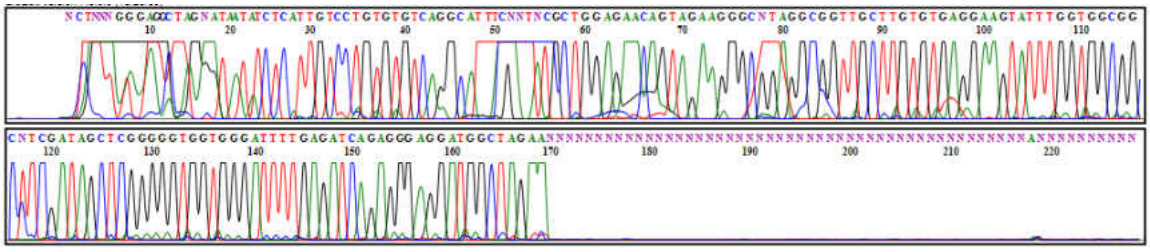
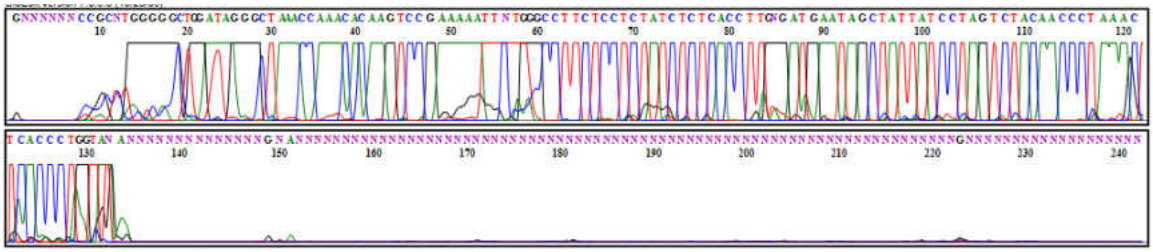
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



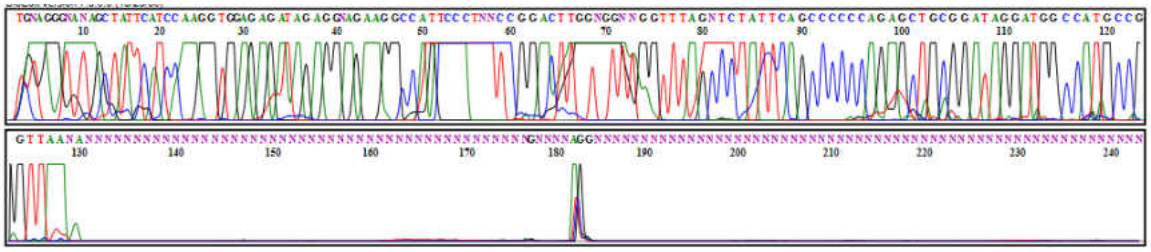
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



Chromatogram for Forward 460-607bp within the mtDNA ND2 gene.



Chromatogram for Reverse 460-607bp within the mtDNA ND2 gene.



Specimen NDSU-2137
MtDNA ND2 Gene Sequence bases 1-1042

----------CTAGCCATCCTCCCTCTGATCTCAAAA

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCTCATCCTAACCTCA----------

----------TAACCGGCATGGCCATCCTATCC

GCAGCTCTGGGGGGCTGAATAGGGCTAAACCAAACACAAGTCCGAAAAATCCTGGCCTTCTCCTCTATC

TCCCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCCTGTTAAACTTCTACCTA

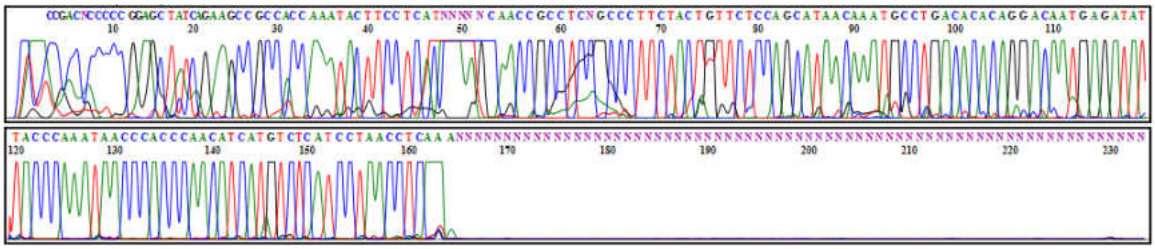
TACACCCTAATAACTGCAGCCATCTTCCTCACCCTAAACTCAACCAACACCACAAAACTATCTACCCTA

ATAACTTCATGAACCAAAGCCCCTGCACTAA----------CCCTACTATCCCTAGCAGGC

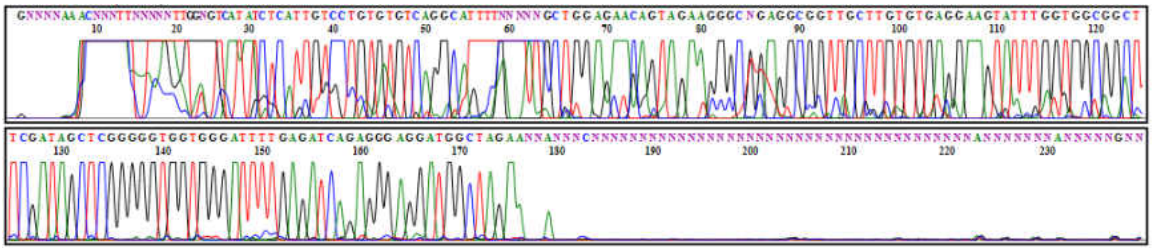
CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGA-ATAACT

CCCGCAGCAACTATCATTGC----------

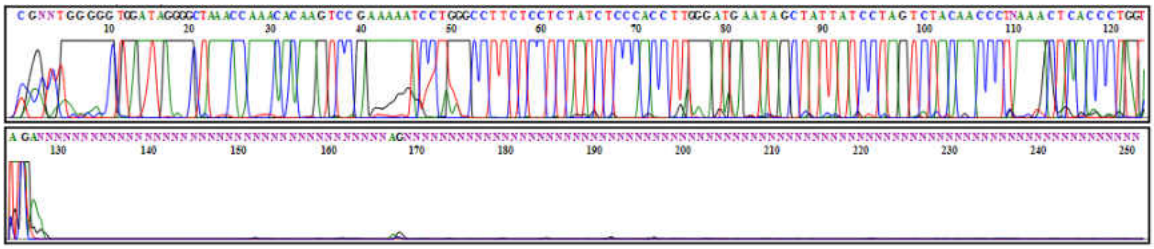
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



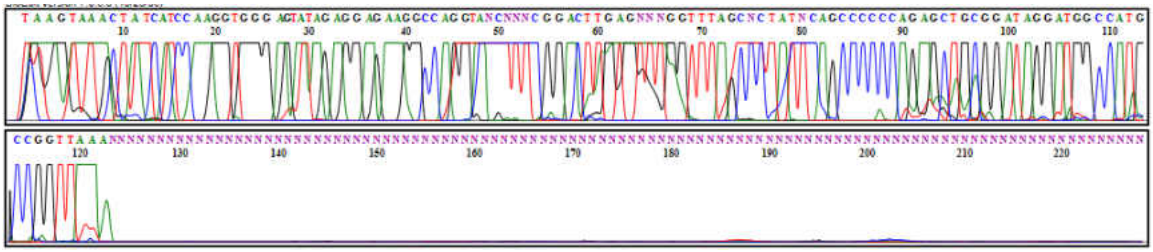
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



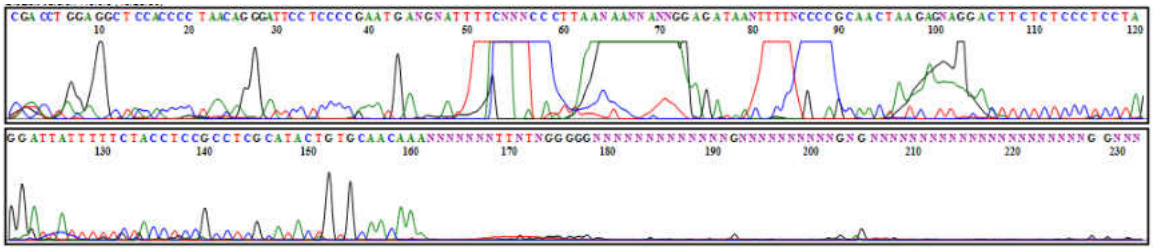
Chromatogram for Forward 460-607bp within the mtDNA ND2 gene.



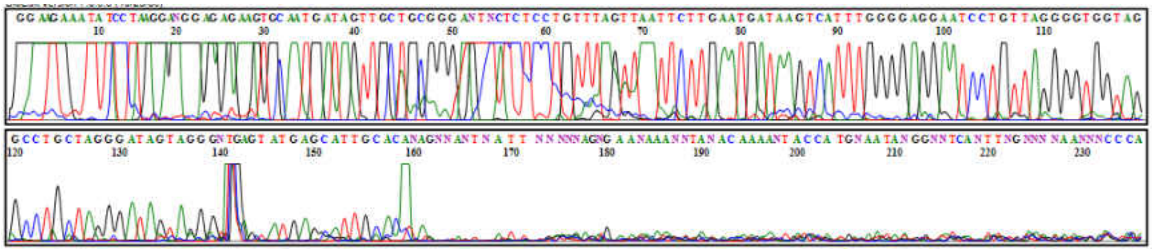
Chromatogram for Reverse 460-607bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



Specimen NDSU-2136
MtDNA ND2 Gene Sequence bases 1-1042

----------CTCTGATCTCAAA-

TCCCACCACCCCCGAGCTATCGAAGCCGCCACCAAATACTTCCTCACACAAGCAACCGCCTCCGCCCTT

CTACTGTTCTCCAGCATAACAAATGCCTGACACACAGGACAATGAGATATTACCCAAATAACCCACCCA

ACATCATGTCTCATCCTAACCTCA----------

----------TAACCGGCATGGCCATCCTATCC

GCAGCTCTGGGGGGCTGAATAGGGCTAAACCAAACACAAGTCCGAAAAATTCTGGCCTTCTCCTCTATC

TCTCACCTTGGATGAATAGCTATTATCCTAGTCTACAACCCTAAACTCACCCTG----------

----

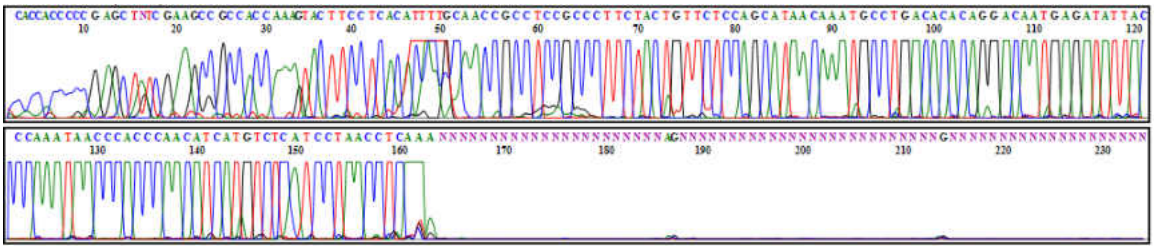
----------TGCAATGCTCATACTAACCCTACTATCCCTAGCAGGC

CTACCACCCCTAACAGGATTCCTCCCCAAATGACTTATCATTCAAGAACTAACTAAACAGGAAATAACT

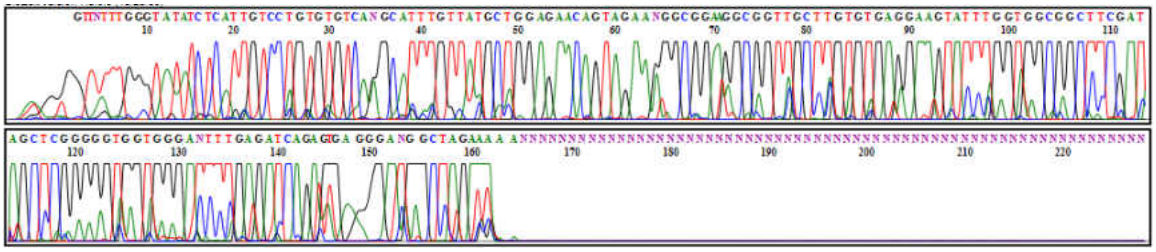
CCCGCAGCAACTATCATTGCACTTCTCTCCCTCCTAGGATTATTTTTCTACCTCCGCCTCGCATACTGT

GCAACA----------

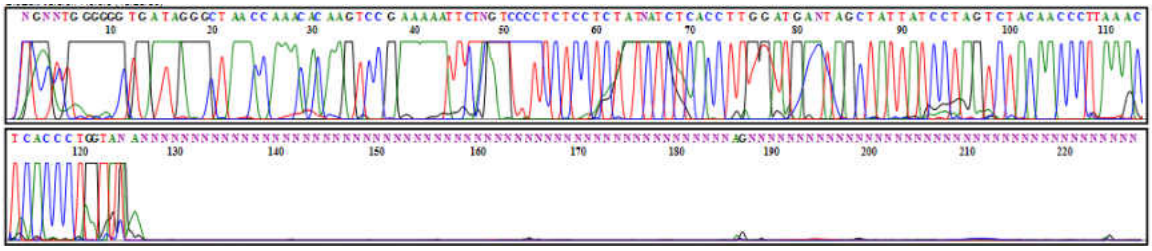
Chromatogram for Forward 110-300bp within the mtDNA ND2 gene.



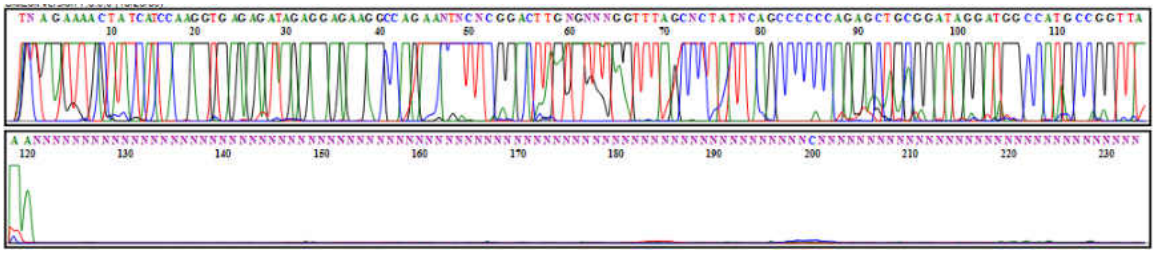
Chromatogram for Reverse 110-300bp within the mtDNA ND2 gene.



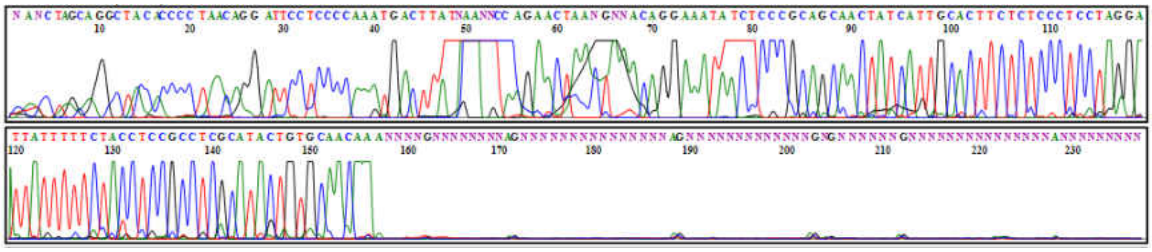
Chromatogram for Forward 460-607bp within the mtDNA ND2 gene.



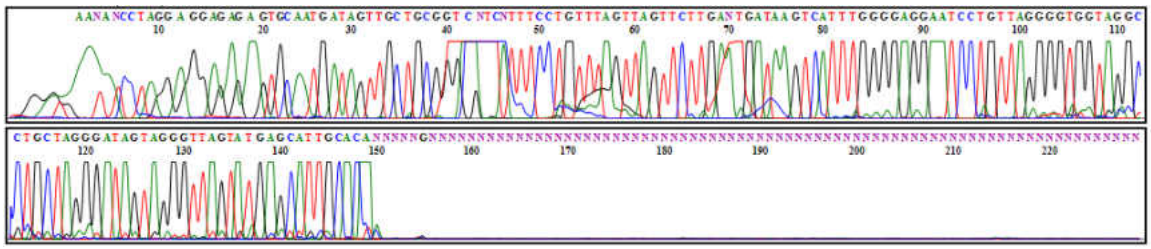
Chromatogram for Reverse 460-607bp within the mtDNA ND2 gene.



Chromatogram for Forward 772-903bp within the mtDNA ND2 gene.



Chromatogram for Reverse 722-903bp within the mtDNA ND2 gene.



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