THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

ANALYZING MITOCHONDRIAL GENETIC DIVERSITY AND DNA DAMAGE IN ANCIENT PASSENGER PIGEON SPECIMENS FROM MUSEUMS

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Spring 2011

A thesis
submitted in partial fulfillment
of the requirements
for baccalaureate degrees
in Science, Spanish, and International Studies
with honors in Biology

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Abstract

Until its extinction in the early 1900s, the passenger pigeon (*Ectopistes migratorius*) was the most abundant species of bird in North America. DNA sequences isolated from museum specimens may help to distinguish between the many hypotheses of its extinction. To this end, ten ancient passenger pigeon samples were obtained from the Royal Ontario Museum in Toronto, Canada to see whether this collection's specimens contained viable DNA, to assess the level of DNA damage in the specimens, and to perform a preliminary assessment of genetic diversity in the passenger pigeon. DNA was successfully extracted, amplified, and sequenced from nine of the ten samples. DNA damage was found in seven of these samples. Phylogenetic and parsimony network analyses showed seven distinct haplotypes among the individuals, suggesting a high level of genetic diversity in the passenger pigeon up until its time of extinction. These results provide a strong basis for proceeding with a larger study to assess the overall population dynamics of the passenger pigeon and to examine the potential causes of the extinction.

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Acknowledgements

Many people are to thank for their assistance in the completion of this honors thesis. All work was carried out in the lab of Dr. Beth Shapiro with the guidance of Dr. Tara Fulton, Dr. Mathias Stiller, and Brandon Letts all of whom offered countless help and guidance throughout this project's duration. Dr. Fulton was extremely helpful in every aspect of this thesis and her assistance is greatly appreciated. Dr. Shapiro is to thank for enabling me to carry out this research. Thanks also to Dr. Stephen Schaeffer for being able to read and approve my final written thesis.

Introduction

A History of the Passenger Pigeon

For centuries before its extinction, observers recorded sightings of passenger pigeons throughout eastern North America. The first recorded observation was on July 1, 1534, and birds were frequently observed until the late 1890's (Schorger, 1973). During this time, no other species of bird on record ever approached the passenger pigeon in numbers. In 1605, there were accounts of "countless and infinite numbers" (Schorger, 1973) of passenger pigeons. Not only were these birds present in high numbers, but single flocks were estimated to have over a billion birds.

While exact population size was not attainable, it has been estimated that upon arrival of Europeans, the passenger pigeon accounted for as much as 40% of the total bird population in the United States, or as many as five trillion individuals (Schorger, 1973). Once Europeans arrived and started to colonize North America, the number of passenger pigeons declined rapidly and significantly. Throughout the 18th and 19th centuries, the number of birds continued to decrease until the species' ultimate demise with the death of the last remaining captive pigeon, named Martha, in 1914 (Wright, 1914). In a matter of 300 years the passenger pigeon was reduced from being perhaps the world's most abundant bird species to extinction, and eyewitness accounts of its persecution by man provide some of the most horrifying descriptions of man's destruction of other creatures (Halliday, 1980).

Many factors have been proposed to explain the passenger pigeon's extinction. First, its demise is often attributed to the purblind greed of hunters who shot the bird in its many millions (Myers, 1987). Bird hunters used nets to capture thousands of birds at a time

(Schorger, 1973). With an increase in hunting and a reproduction rate of only 1 egg per year, the species could not reproduce to counter its killing. Second, disease has been proposed as a main contributor to extinction. Many pigeons in captivity were susceptible to trichomoniasis, more commonly know as canker, which was spread from domestic pigeons to passenger pigeons (Schorger, 1973). Exotic tropical diseases were also attributed to various pigeon deaths. One researcher observed that in a room of 1,000 confined passenger pigeons, all were dead after their morning feeding, giving credibility to the disease hypothesis (Schorger, 1973). Another popular extinction hypothesis is that the deforestation of North America caused the species to nest further and further northward. With this move, harsh weather conditions made it difficult to mate and raise the young (Schorger, 1973). The flocks consisted of older birds that became weaker year after year with the onset of ice and snow.

No matter what hypothesis one uses to describe passenger pigeon extinction, the species could not exist in small numbers, rather it needed large flocks in order to survive. Once the flocks were fragmented, they could not maintain stability to keep the species going. A researcher wrote, "The pigeon, like the buffalo, was a species whose existence seems to have depended upon association in large numbers and once separated and scattered into small flocks and pairs its doom was sealed" (Schorger, 1973).

Although there is no clear answer as to why the passenger pigeon went extinct, it is now possible to use genetics to attain some answers. Through advances in DNA extraction and sequencing, samples of preserved remains can be genetically tested to assess past genetic diversity. By gathering a large, temporally and geographically diverse sample of specimens, one can observe differences in diversity over time and space. Using this

approach it is possible to test hypotheses about declines in genetic diversity over time and across space.

Ancient DNA

The use of ancient DNA (aDNA) is fairly new, with the first successful ancient DNA extraction taking place in 1984 by Russ Higuchi. In this work, Higuchi and colleagues were able to obtain mitochondrial DNA from muscle sampled from a museum specimen of the now-extinct quagga, a member of the horse family. Due to the low amount of template DNA in the ancient sample, only 229 base-pairs (bp) of DNA were successfully sequenced (Higuchi, 1984). Even with this small fragment, it was shown that it is possible to obtain DNA from ancient samples and test hypotheses about the relationships of extinct animals to extant taxa.

With advances in technology in molecular biology, most notably the invention of the polymerase chain reaction (PCR) in 1983 (Mullis, 1987), aDNA research has become a useful tool to look at species of the past. In a normal PCR, a template strand of DNA is copied through various cycles of annealing, replication, and elongation. When amplifying modern DNA, PCR is fairly simple, as the template strand of DNA is often undamaged. However, with aDNA, it is very rare to have a perfect template strand, making ancient PCR a difficult task.

Base modification of ancient DNA occurs by fragmentation via strand breaks the accumulation of damage lesions, such as baseless sites, oxidized pyrimidines, and cross-links (Pääbo, 1989). These modifications are so extensive that less than 1% of the DNA molecules extracted from museum specimens or archaeological finds can be expected to be undamaged (Pääbo, 1989).

This experiment targeted mitochondrial DNA, as opposed to nuclear DNA, for various reasons. Primarily, mitochondrial DNA is present in a much higher copy number per cell than nuclear DNA, making it more likely to survive through time. Mitochondrial DNA also is useful for this study as it has regions that show great variation due to a higher mutation rate (Brown, 1979). The highly variable mitochondrial d-loop was used and amplified in the obtained samples.

In addition to damage, contamination is also a challenge in successfully extracting, amplifying, and sequencing ancient DNA. The enormous amplifying power of PCR has created an increased sensitivity to contamination from modern DNA, and simultaneously, a major potential source of such contamination through the extraordinary concentrations of previously amplified PCR products (Willerslev, 2005). With this in mind, it is necessary to follow appropriate contamination prevention techniques to ensure the authenticity of any data obtained. Such techniques include performing all DNA extractions and PCR set-up in a clean room located in a building that is separate from modern molecular biology work. This helps ensure that only ancient DNA is amplified rather than a modern contaminant.

The goal of this study was to examine the possibility of using genetic methods to study the decline of the passenger pigeon. I amplified the d-loop from mitochondrial DNA of museum specimens of 10 passenger pigeon aging up to 130 years old. Specifically, I assessed whether these specimens had undamaged DNA, varied genetically, assessed the level of DNA damage, and identified any correlations that may exist between age of specimen and DNA damage.

Materials and Methods

DNA Extraction

Passenger pigeon toe pads were collected from the Royal Ontario Museum in Toronto, Canada (Table 1). DNA extraction and amplification was performed in a dedicated aDNA facility at The Pennsylvania State University that is housed in a building separate from where genetic analysis were performed. Ancient DNA protocols were strictly adhered to at all stages (Cooper and Poinar, 2000; Gilbert et al., 2005). An extraction negative was used to verify absence of contamination. Prior to extraction, each individual toe pad was cut into small pieces. DNA extraction was carried out using the Qiagen DNeasy Tissue Kit (Qiagen) following manufacturer's instructions with an extended lysis of 48 hours using 20 µl proteinase K and 20µl of 1M DTT. An additional 20 µl of proteinase K was added after the first 24 hours. The samples were rotated and heated to ensure proper mixing and distribution of reagents. After the samples were completed dissolved, the extraction solutions were transferred to DNeasy spin columns and manufacturer instructions were followed to elute the DNA. The final elution was performed using 50 µl of AE buffer.

Table 1. Passenger pigeon sample information collected from the Royal Ontario Museum

Sample #	PSU Accession #	Royal Ontario Museum Accession #	Location	Date
BL241	UP10.TF381	40356	Wayne Co., Michigan	1898
BL242	UP10.TF383	33.6.20.3	Peterborough Co., Ontario	1891
BL243	UP10.TF380	40359	Montreal, Quebec	1888
BL244	UP10.TF379	26.11.1.1	Hastings Co., Ontario	1890
BL245	UP10.TF384	67037	Hamilton, Ontario	1863
BL246	UP10.TF382	34.5.8.21	Lake Co., Indiana	1888
BL247	UP10.TF386	29150	Hamilton, Ontario	1863
BL248	UP10.TF385	76856	Toronto, Ontario	1860
BL249	UP10.TF387	34.5.17.1	Oxford-Brant, Ontario	1863
BL250	UP10.TF388	33501	Niagara Falls, Ontario	1860

Primer Design

The mitochondrial d-loop was chosen for amplification due to its high genetic diversity and variability (Brown, 1979). Using previously sequenced d-loop sequences from *Columba inornata* (Young, 1997), primers were designed to amplify two overlapping fragments of approximately 140 bp each, for a total of 259 bp. Primer design was carried out using software available as part of the DNAStar package (DNASTAR, Inc.) (Table 2).

Table 2. Primer sets designed to amplify selected segments of mitochondrial d-loop region

Primer Name	5'-3' Sequence	Annealing Temperature
Emig.DL-F1	ACACAGCCATCCTTCCAGAG	57°C
Emig.DL-R1	ACCTGCTTTGTGTACTTCAG	57°C
Emig.DL-F2	CCAAAACAACACGGAAGTGC	53°C
Emig.DL-R2	AAGTCGTGCTAGGGTGTAGG	53°C

DNA Amplification

PCR amplifications were carried out in 25 µl reactions consisting of 5µl of 50µg rabbit serum albumin, 2.5 µl of 0.25mM dNTPs, 2.5 µl of 10X High Fidelity buffer, 0.25 µl Platinum *Taq* High Fidelity (Invitrogen), 1 µl of 3mM $MgSO_4$, 1 µl of each primer, and 1µl DNA extract**.** Cycling conditions were 94°C for 2 min., 55 cycles of 94°C for 30 sec., 45 sec 57°C (F1)/53°C (F2), 68°C for 45 sec. followed by 45 sec. of 68°C. Negative PCR reactions not containing DNA were run with each set of PCRs.

The PCR products were visualized under UV light using ethidium bromide after resolution on a 2% agarose gel. Amplification was deemed successful when the correct size band was visualized and there was no similarly sized band in the PCR negative.

Successful reactions were cleaned to remove any unwanted and unincorporated

products by adding 80 µl of autoclaved H_2O to each reaction. The entire solution was then transferred to a Millipore filter plate. A vacuum was used to remove all the water. After approximately 10 minutes, when all the water was removed, 50 µl of H_2O was added and the vacuum process was repeated. After 10 additional minutes, 30 µl of H_2O was added to each reaction. To resuspend the DNA, the filter plate was shaken for 30 minutes.

Cloning

To analyze DNA damage and assess contamination, each amplified individual was cloned using a TOPO TA cloning Kit (Invitrogen). Each cloning reaction was set up using 0.25 µl TOPO vector, 0.25 µl salt solution, 1.5 µl H_2O, and 1 µl cleaned PCR product. The manufacturer's instructions were followed for the remaining steps. Plates were incubated at 37 °C overnight. After incubation, 8 white colonies were picked from each cloned reaction. The picked colonies were placed in 50 µl of H_2O. A 12.5 µl cloning PCR reaction was prepared with 5 µl vortexed colony in H_2O, 1.25 µl 10X buffer, 0.125 µl Econo-Taq, 0.125 µl 25mM dNTPs, 2.25 µl H_2O, 1.25 µl 25mM $MgCl_2$, and 1.25 µl of both primer sets M13F and M13R. Cycling conditions were 94°C for 2 minutes, 30 cycles of 94°C for 1 minute, 1 minute 56°C, 72°C for 1 minute followed by an extension of 7 minutes at 72°C.

Cleanup and Sequencing

Cloned PCR reactions were cleaned using an Ethanol/EDTA precipitation procedure. First, 2.5 µl of 125 mM EDTA and 25 µl of 100% EtOH were added to each reaction well. The reaction plate was incubated for 15 minutes at room temperature and then centrifuged for 30 minutes at 2750 x g .Immediately after centrifugation, the plate was turned upside down onto a properly sized paper towel. The plate was then centrifuged for 2 minutes at 500rpm. After, 30 µl of 70% EtOH was added to each well. The plate was returned to the

centrifuge for 10 minutes at 2750rpm. As before, after spinning completed, the plate was inverted onto a paper towel and spun at 500rpm for 2 minutes. The plate was then transferred to a dark drawer to allow overnight evaporation of the 70% ethanol. After evaporation, 20 µl of H_2O was added to resuspend the DNA. 10 µl of the reaction was sent to the Penn State Genomics Core Facility to obtain sequences using an ABI3730xl DNA Analyzer.

Analysis

Sequences were by eye aligned using Se-Al v2.0a11 (http://tree.bio.ed.ac.uk/software/seal/) to observe damage and mutations. Neighbor joining trees and maximum parsimony bootstrap trees with 1000 replicates were constructed using Paup* v.4.0b10 (Swofford, 2003). In addition to phylogenetic trees, a network was constructed using the program Network 4.6.0.0 (Bandelt *et al.*, 1999) to observe relationships between individuals.

Results

DNA was successfully extracted from 9 of the 10 ancient samples obtained. Of those, fragment 1 was successfully sequenced in all nine individuals. Sequences were obtained for fragment 2 in four of the samples.

Assessing aDNA Damage

By visualizing the aligned clone sequences using Se-Al v2.0a11 (http://tree.bio.ed.ac.uk/software/seal/), it is possible to view where substitutions occurred to observe areas of damage in the DNA strand stemming from differently damaged template molecules.. Theoretically, being clones, all the sequences should be exactly the same without alteration. However, because the cloned PCR products may be

amplified from different starting molecules, it is possible to characterize the amount of damage in the sample in this manner. In addition to detecting DNA damage, this procedure is useful to infer the correct consensus sequence, which may be masked by damaged bases in direct sequences of amplified PCR products.

Table 3. Number of bases per sample showing DNA damage after cloning

Sample #	Number of Damaged Bases	Date
BL241	1/147	1898
BL242	2/147	1891
BL243	3/259	1888
BL244	3/259	1890
BL245	6/259	1863
BL246	1/147	1888
BL248	0/259	1860
BL249	2/147	1863
BL250	0/147	1860

Table 4. Specific base substitution types in all individuals

Sample #	Damaged Base	Observed Substitution	Ratio	Fragment(s)
BL241	#131	G-A	6G:1A	F1
BL242	#32	C-T	2C:1T	F1
BL242	#111	C-T	2C:1T	F1
BL243	#91	C-T	5C:1T	F1
BL243	#126	C-T	11C:2T	F1
BL243	#130	C-T	12C:1T	F2
BL243	#133	C-T	12C:1T	F2
BL244	#134	C-T	12C:1T	F2
BL244	#146	C-T	6C:1T	F2
BL244	#163	A-G	6A:1G	F2
BL244	#176	T-C	6T:1C	F2
BL244	#257	C-T	4C:3T	F2
BL245	#27	C-T	5C:1T	F1
BL245	#42	T-C	9T:2C	F1
BL245	#82	C-T	9C:1T	F1
BL245	#103	T-C	13T:1C	F1, F2
BL245	#116	T-C	10T:7C	F1, F2
BL245	#130	C-T	15C:2T	F1
BL246	#132	T-C	6T:1C	F1
BL249	#85	A-G	7A:1G	F1
BL249	#142	C-T	6C:2T	F1

Phylogenetic Analysis

Of the 9 samples from which d-loop sequences were successfully sequenced, 259 total bp were obtained in 4 of the 9 individuals. For the 5 samples where only fragment 1 was sequenced, 147 bp were obtained. Of the total 259 bp amplified using both fragments, 251 were invariable. Only 2 of 8 variable sites were parsimony informative. All 8 variable sites are contained within fragment 1. Fragment 2 is invariant at all nucleotides. Table 5 lists the haplotypes observed and their respective variable sites. Figure 1 showing the geographic distribution of the haplotypes.

Table 5. Haplotypes found among tested individuals and their respective variable sites

		Variable Sites							
Haplotype	Individuals	32	42	60	77	87	103	111	143
1	241, 250	C	C	T	T	T	C	T	T
2	243, 246	•	•	C	•	•	•	•	•
3	242	•	•	C	•	•	•	C	•
4	244	T	•	C	•	•	•	C	•
5	245	•	T	•	•	A	T	•	•
6	248	•	•	C	C	•	•	C	•
7	249	•	•	C	•	•	•	C	C

Figure 1. Geographic distribution of haplotypes

Neighbor joining analysis (Figure 2) grouped individuals BL249, BL244, and BL242 together in a single clade. . This clade was weakly supported by maximum parsimony bootstrapping (MPBP = 64%). No other clade was supported in the MP bootstrap analysis.

Network analysis (Figure 3) also recovered the same pattern grouping individuals BL249, BL244, and BL242 together.

Figure 2. Unrooted neighbor joining tree constructed using paup

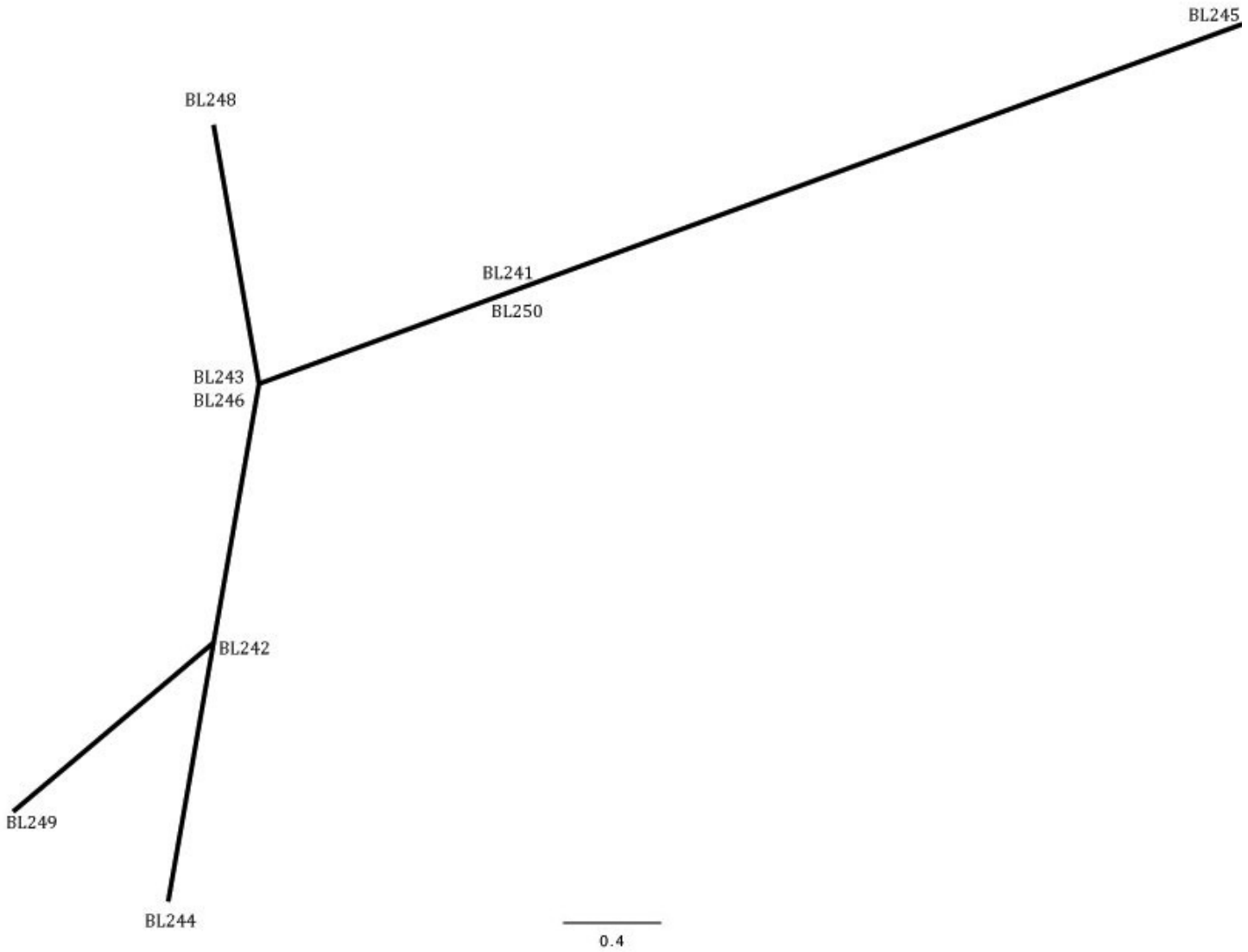
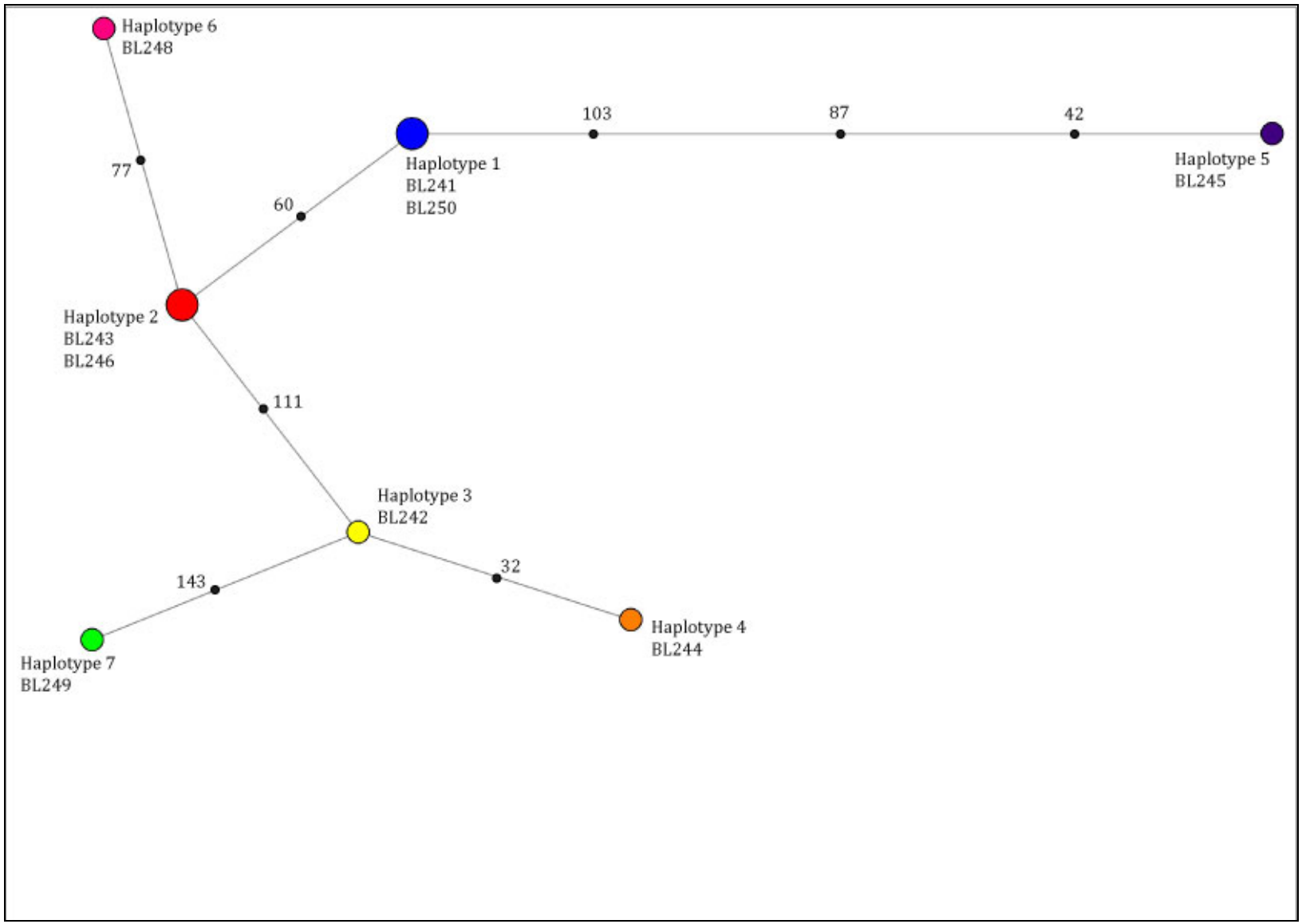


Figure 3. Parsimony network of all haplotypes showing specific mutations



Discussion

DNA Damage

Most samples showed some signs of DNA damage. One sample, BL247, did not contain any DNA after extraction and repeated amplifications. Seven of the nine samples that contained DNA had at least one damaged base (Table 3). One likely hypothesis for the cause of damage is related to the age of the sample, where older samples should show more damage than younger samples. However, we find the opposite of this: the oldest samples (BL248 and BL250), both dating back to 1860, had no observed damaged bases. Two explanations for this are that all the samples come from the same museum and had therefore been subjected to the same preservation conditions, and that there is only a small (40-year) age difference between the oldest and youngest sample. It is hypothesized that age would be more of a factor with a greater gap in time.

A majority of the damaged bases consisted of C/G-T/A transitions (Table 4), which have been previously explained by researchers as an effect of the deamination of cytosine residues (Stiller, 2006). The other observed changes in these museum samples were T/A-C/G. There are two likely explanations for this. First, even though the consensus sequence suggests that the true sequence at that site is a T, based on previous findings, the occurred damage is more likely a C-T transition, not a T-C. (Hofreiter, 2001). This is most likely the case for the damage at base #42 of BL245, where 2 C's are observed among different clones at the same base. In other cases, where only 1 C and the rest T are observed amongst different clones, as in base #132 of individual BL246, the more probable cause for this transition could be attributed to an error made by the DNA polymerase (Platinum *Taq* High Fidelity) used in this experiment. A previous study compared this polymerase to another

commonly used polymerase (AmpliTaqGold) where it was fond that Platinum *Taq* High Fidelity misincorportaed T for C 10 times more often than AmpliTaqGold (Stiller, 2006). This elevated tendency for substitution could explain the T/A-C/G transitions observed in this study.

Genetic Diversity

Seven distinct haplotypes were found among the 9 individuals tested. Individuals BL241 and BL250 share haplotype 1 (Table 5), however, these samples were separated by a substantial geographic distance (Figure 1). They also were very different in age, with BL241 dating back to 1898 and BL250 dating back to 1860.

Haplotype 2 was also observed twice, in individuals BL243 and BL246. As with haplotype 1, these two individuals varied geographically, but both samples were collected in 1888. Although the exact month of collection is unknown, it can be hypothesized that due to the migration patterns of the passenger pigeon, that these two individuals could be from the same flock.

The neighbor joining tree (Figure 2) shows a weakly-supported clade comprising BL249, BL244, and BL242. This tree looks identical to the constructed network of the individuals (Figure 3). Sample BL245 (Haplotype 5) stands out as the most genetically distinct of the 9 samples. BL245 is distinct from BL241 and BL250 (Haplotype 1) by three differences at bases 42, 87, and 103.

Further Areas of Research

The initial purpose and goal of this study was to assess the population genetics of the passenger pigeon over time and space. To successfully carry out a study of that caliber, a large sampling size is needed. The passenger pigeon is an extinct species, and museums

are understandably reluctant to offer their specimens for destructive sampling. Thankfully, for this pilot study, the Royal Ontario Museum offered 10 individuals from their collections. Based on these results, after showing that DNA was successfully extracted and sequenced, a future study with a greater quantity of samples is likely to be supported.

With a greater sample size, a larger study on the population history of the passenger pigeon could be conducted. It is possible to observe the overall genetic diversity of the passenger pigeon both geographically and temporally and to assess a possible cause of extinction. Microsatellites, which are more variable than mitochondrial d-loop sequences (Ellegren, 2004), could also be targeted to characterize changes in population structure over time. By targeting these highly variable regions, it will be possible to test the alternate hypotheses proposed to explain the extinction of the passenger pigeon.

Conclusions

This research project analyzed both the genetic diversity of the passenger pigeon and damage to DNA in museum-preserved specimens of pigeon toe pads. The study was successful in the extraction, amplification, and sequencing of DNA from nine of ten ancient samples tested, indicating good DNA preservation. However, DNA damage was present in the majority of specimens. In an amplified region of mitochondrial d-loop of passenger pigeon specimens, diversity was shown and distinct haplotypes were observed among the samples. However, there was no correlation between diversity and either time or geographic distribution of these samples. Overall, this study showed that future, larger scale projects involving the population genetics of the passenger pigeon will be able to be carried out.

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- Managed a database for patients who underwent a Gynecare Prolift® procedure to repair vaginal vault prolapse
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- Utilize effective problem solving skills to provide quality customer service

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PUBLICATIONS

N.Bhatia, M Murphy, V. Lucente, N. Ehsani, H. Devakumar, **R. Shaw** and S. DeTurk. *A Comparison of Short Term Sexual function Outcomes for Patients Undergoing the Transvaginal Mesh Procedure Using the Standard Polypropylene Mesh vs. a hybrid POLYPROPYLENE/POLIGLECAPRONE Mesh, Oral* Poster, Journal of Female Pelvic Medicine & Reconstructive Surgery, Volume 16, Number 2, March/April 2010.

UNDERGRADUATE RESEARCH

Honors Thesis: "Analyzing Mitochondrial Genetic Diversity and DNA Damage in Ancient Passenger Pigeon Specimens from Museums"
Advisor: Beth Shapiro, D.Phil.

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Phi Beta Kappa- inducted Spring 2010
Dean's List- all semesters
Ruth Duffy Premedicine Endowment: $2,000

ACTIVITIES

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Internal Relations Adviser, Fall 2010-present
Membership Committee, Fall 2009-Spring 2010
Undergraduate Admissions Tour Guide, Fall 2008-present

- Provide several tours per month for Penn State's Office of Undergraduate Admissions
- Act as a representative of Penn State and encourage prospective students to attend
- Serve as a member of the Executive Board

Alpha Epsilon Delta (AED), National Premedical Society
Honors Member, Fall 2008-present

- Raise money to support Penn State's Pan-Hellenic Dance Marathon (THON)
- Learn about health careers through speeches by various professionals

Eberly College of Science LionPride
College of Science Student Ambassador, Spring 2009-Fall 2010

- Assist the Eberly College of Science recruitment office in seeking prospective students and helping those students choose to attend Penn State.

Bronx Aerospace High School Pen Pal Mentor, Fall 2008-Spring 2009

- Corresponded monthly with a pen pal in an underprivileged city school motivating him to attend college

Introductory Biology Tutor, Fall 2009

- Tutored freshmen Biology majors in the introductory course and laboratory

SKILLS

Language: Fluent in Spanish
Computer: Knowledgeable in Microsoft Word, Excel, PowerPoint, SPSS/PASW Statistics