UH 450 Honors Thesis:

Feasibility of Mitochondrial DNA Isolation from Ancient Fish Bones

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Honors Senior Thesis
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PASS WITH DISTINCTION
Feasibility of Mitochondrial DNA Isolation from Ancient Fish Bones

Damon Scoville

Though we all wonder what the future holds, many scientists focus on the past and concentrate on how the human race has arrived where we are today. But it is not only the Homo sapiens's past that is studied. Scientists have long observed living beings around them and sought answers for 'what is this organism?', 'why is it here?', 'what is its purpose?', and 'how did it come to be?'. Archaeologists toil with minute fragments of ancient bones to elucidate an organism’s species and period of existence. Scientists now ponder questions concerning which creatures are related to one another in the present and which are related or derived from organisms identified from the past. Though bone shape and structure, carbon dating, and the location of bones have given strong indications and hints as to ancestral heritage, only by examining a being’s DNA can we truly be sure about actual identity.

Within the scientific community, one of today’s most controversial, exciting, and arduous topics lies in the field of ‘ancient DNA’ (aDNA). With the advent of the Polymerase Chain Reaction (Mullis, 1987), or PCR, it has become possible, in theory, to amplify fragments of DNA starting from just one copy of the target DNA. Utilizing PCR, scientists have been able to isolate, amplify, and sequence DNA from various fossils dating from tens of thousands of years ago. Using known sequences from present day organisms, scientists have gleaned information about where organisms originated and traveled to, which organisms show signs of evolutionary relatedness to one another, mutation frequencies, and even when certain diseases may have become prominent in the past. Especially important today is the subject of ancestral origins and relatedness to present day beings. Many Native Americans struggle against scientists to protect ancient human remains of beings they believe to be their ancestors, whom they want to protect from desecration. On the other side of that issue, anthropologists want to use aDNA technology to prove the actual origins of the ancient bones. One cannot be absolutely sure of relatedness until actual tests are performed, and that requires a small portion of bone for DNA extraction.
Another controversial aspect of aDNA technology lies in the validity of the work itself. There are those who believe that contamination, or DNA from the present, is the only DNA being amplified in aDNA experiments. This is, unfortunately, a real concern. PCR may theoretically amplify from just one copy of DNA. Therefore, if even one molecule of contaminating DNA gets into the PCR mixture, it too can be amplified along with any other DNA already in the reaction. "...Minute amounts of contaminating DNA may be preferentially amplified, especially when the ancient extract contains few or no endogenous DNA molecules, and contaminating DNA could out-compete contaminating DNA during PCR because it is usually of more recent origin and therefore, less damaged" (Kumar, 1999). Obviously, steps need to be taken to ensure that no contamination corrupts any PCR reaction.

So, what are possible sources of aDNA? One might be surprised to find there are more than one might first think. The obvious first choice is fossilized bone. A majority of aDNA research has concentrated on extracting nucleic acids from bones ranging in age from the present day to hundreds of thousands of years ago. Besides fossilized bone, archaeologists also find other media that may contain DNA. Amber has trapped many unsuspecting insects that have been eventually preserved for long periods of time. "The list of amber preserved specimens from which ancient sequences were recovered is impressive and includes stingless bees, termites, honeybees, and plants"(Wayne, 1999). Fossilized dung from various animals has also been utilized to recover DNA. Owl droppings many times contain full or nearly full skeletons of an owl’s previous hunt, and sequence has been salvaged this way (Wayne, 1999). Museum skins are yet another source of aDNA (Wayne, 1999), but these obviously lack the antiquity of fossils. Plant sequences have also been rescued. Finally, fossilized bark or leaves is a media from which aDNA is salvageable (Dumolin-Lapegue, 1999).

Finding these sources of ancient DNA can be a challenge. Though fossils have been found all over the world in any number of climate zones, preservation is the key. The right conditions are essential to the recovery of aDNA. Cold, dry conditions are best. Places such as caves in arid locales and the permafrost regions in Siberia have provided fossils with well-preserved aDNA. The most compelling reason for sample preservation in these conditions is bacterial growth is usually restricted in these types of areas.
Hypersaline pools, acidic moorlands, high altitudes, and temperate soils have also yielded fossils with recoverable DNA. Moreover, the Rancho La Brea tar pits trapped thousands of specimens over the years and sequence from a saber-toothed cat, *Smilodon fatalis*, were rescued recently. (Wayne, 1999)

Along with preservation goes time. There is a limit to the amount of time DNA can stay intact, even under the best of conditions. “Theoretical calculations have suggested that DNA may not survive for more than 10,000--100,000 years, and even if it does it is expected to be highly fragmented and chemically modified” (Kumar, 1999). One hundred thousand years is a very minute amount of time when thinking about fossils. Dinosaur remains have been found to be millions of years old, so the chances of recovering actual dinosaur sequence are incredibly low. Claims have been made that DNA from million year old fossils was recovered, although later experiments by other laboratories showed that the experiment was either irreproducible, the DNA recovered was an artifact, or the DNA recovered was due to contamination (Kolman, 2000; Wayne, 1999; Kumar, 1999).

DNA may be preserved, in theory, for up to one hundred thousand years. But, this does not mean that all DNA in a cell is preserved for that long. In fact, genomic DNA, being linear, doesn’t survive very long at all. For most aDNA experiments, the researcher will be concentrating on looking at mitochondrial DNA (mtDNA), located in the mitochondria of eukaryotes. There are three very compelling reasons to employ mtDNA in aDNA experiments. First, eukaryotes contain sometimes hundreds of mitochondria per cell, and each mitochondrion contains many copies of its mtDNA, usually around thirty copies (Lodish, 2000). This translates to upwards of thousands of copies of an organism's mtDNA per cell. But, not only is mtDNA plentiful. It is also more stable than genomic, or chromosomal, DNA. Mitochondrial DNA is small and circular, lending stability. Moreover, mtDNA also contains some very highly conserved sequences. These sequences are specific enough in some places to vary by only a few base pairs between closely related species. Many times this makes placing an organism into a particular group very easy. This experiment utilized portions of the mitochondrial control region to elucidate the identities of the fossil samples.
DNA has been isolated from many mammals. In mammals, the organisms' stem cells, which are the cells that produce more cells and are therefore active and harbor many mitochondria, are located in the marrow, on the inside of the bone. Fish, on the other hand, do not grow this way. Fish bones grow outward in concentric circles; imagine the rings in a tree. The active layer of stem cells in fish bone is on the outside. This presents a problem. When dealing with preservation, mammalian bones are ideal because the stem cells are protected on the inside, and are better able to be preserved through weathering and changing conditions. With fish bones, the stem cells are the first to go. This makes extracting DNA more difficult because one has to hope the rest of the bone will yield enough DNA.

Another problem with some preserved fish bone samples is the size. In the case of this experiment, bigger is definitely better, yet many of the samples were very small, further lowering the chances that good DNA would be isolated.

Experimental:

The goal of this research was to speciate the 2,600-year-old fish fossils found at a dig site on the Owyhee River, in Malheur County, in southern Oregon State. They were presumed to be salmonid and one of three species: Sockeye Salmon (*Oncorhynchus nerka*), Chinook salmon (*Oncorhynchus tshawytscha*), or Rainbow Trout (*Oncorhynchus mykiss*), this based on known species in the river now, and on other records. Nested primers were constructed from a highly conserved portion of the mitochondrial genome, specifically in a region where sequence from the three species listed above could be used to differentiate between the three. The outer primers (ANPO-R1 and ANPO-F1) are approximately 389bp apart and the inner primers approximately 230bp apart. The primers amplify a conserved region starting at the 3' end of the cytochrome B sequence through the threonine tRNA sequence, the proline tRNA sequence, and into the 5' end of the mitochondrial control region. The four primers we constructed are listed below:

ANPO-F1 5'-AGGTTAAACCCTCCCTAGTGCTC-3'
ANPO-R1 5'-AAAGTTGGTGGGTAAGACGGAGCCC-3'
ANPO-F2 5'-TCCACCCCTTAACTCCCAAAGC-3'
ANPO-R2 5'-GGATTTGTGCTGATGTGAGGG-3'
Later, nested primers constructed by another laboratory were used. These primers were constructed from the far end of the mitochondrial control region through the adjacent phenylalanine tRNA sequence and into the 5' beginning of the 12s rRNA sequence. The outer primers are approximately 311bp apart and the inner primers approximately 233bp apart. They are listed below:

- **P-2 Outer**: 5'-TACCCCCCTACGCTGAAAGATC-3'
- **S-Phe Outer**: 5'-TGCTAGCGGGACTTTCTAGGG-3'
- **P-2**: 5'-TGTTAAACCCCTAAACCAG-3'
- **S-Phe**: 5'-GCTTTAGTTAAGCTAC-3'

Both sets of 4 primers were tested on positive control samples from all three salmonid species. Two known tissue samples from each of the three species were used to perform DNA extractions using the PureGene® DNA Isolation Kit D-5000A from Gentra Systems. PCR was run using every possible combination of each set of the ANPO primers on all three species. All were successful, though a band was observed at approximately 50bp on most of the gels. This was assumed to be a primer dimer band. It was the right size and was a probable possibility concurrent with the extra high amount of primers used in the PCR protocol adapted for use with mitochondrial DNA. DNA sequencing of the PCR products for each of the three species was also performed. Sequence obtained for each species matched known present day sequences (Shedlock, 1992 and Zardoya, 1995).

Next, bone samples, specifically vertebrae, from present day specimens were obtained from our hatchery. A vertebra was taken from a large and a small specimen, stripped of tissue, boiled for approximately 10 minutes, and dried for a period of 7 days at 37 C. The bones were then used as the ancient samples would be and were subjected to the DNA extraction protocol. From there, PCR was performed on the two samples using both sets of primers. All were successful. Thus, mtDNA could be extracted from bones and be amplified by PCR.

Soon after, 8 ancient samples were delivered and DNA extractions were performed. PCR using the outer ANPO primers was subsequently carried out on all 8 samples, with a positive control (a sockeye salmon sample). No amplification was
observed, and the positive control showed a smear. After running the same PCR again, the positive controls (samples from all three species) worked, but no ancient samples yielded any amplification. Next, the inner primers were tried, with much better results. Amplification was observed on all but two ancient samples, and on all positive controls. All positive PCR samples were cleaned and sequenced. Sequencing yielded a match to Chinook Salmon sequence, which was unexpected. Also, it was not only one ancient sample that yielded the Chinook sequence, but all ancient samples had Chinook sequence. We suspected something was amiss. Most likely the ancient samples had been contaminated with Chinook DNA.

After further reading on techniques and protocols for ancient DNA research, work was subsequently moved to another lab room down the hall. Here, equipment and reagents were set aside for use only with this project's aDNA research. Also, a benchtop UV sterilizer was obtained to store equipment and reagents in and to perform experiments in. The UV was checked by a BLAK-RAY UV meter from UV Products, Inc. The UV sterilizer was turned on when experiments were not being performed to help sterilize the environment.

More experiments with the ancient samples were carried out, now with negative controls. At first, the negative controls would exhibit bands after PCR, indicating contamination from somewhere. Eventually though, negative controls were seen to be negative, except after 3 or more rounds of PCR. But, there was still no amplification from the ancient samples. We hypothesized that something in the extraction may have been inhibiting PCR in the ancient samples. To fix this, the ancient samples were gene cleaned using a BIO 101 GeneClean III Kit, but still no amplification was observed after PCR. Also, to check on the inhibition hypothesis, ancient samples were 'spiked' with known positive controls and PCR was carried out. PCR was unsuccessful for an unknown reason.

To combat contamination, the GeneCleaned ancient sample extracts, run through PCR once with the inner S-Phe and P-2 primers, were cloned using the pGEM T-Easy cloning system into Escherichia coli library efficient DHSα competent cells from GibcoBRL. By using the entire extract for cloning, this would mean that newer DNA could not out-compete older, degraded DNA. Each fragment of DNA present would
have an equal chance of being transformed into the host cells. Unfortunately, the cloning was unsuccessful.

A final hypothesis concerned the bones themselves. It was suspected from the small size of the fossils that they might not have been salmonid, in which case the primers would not have worked even if DNA were present. Squawfish (*Ptychocheilus oregonensis*), which are unrelated to salmonid species, are common in many rivers and are smaller fish. DNA from Squawfish was obtained and PCR carried out on it. Amplification was unsuccessful, validating the hypothesis that if indeed the bones were not salmonid, and they were suspected to be Squawfish, PCR would not work.

At this point, time was running out, so I decided to stop research and focus on designing a protocol for anyone who would come after me. The following is a plan for how aDNA research on ancient fish bones should be performed to conduct successful experimentation. Basically, this is how to do it the right way, based off of what I learned during my three semesters of research.

**Lab, Equipment, Reagents, and Storage:**

There should be no other research being conducted in the laboratory room where the aDNA research is being performed. Also, to be safe, aDNA research should be conducted in a building separate from one that conducts research on the same organisms being studied. On top of that, positive and negative controls should also be performed in different parts of the lab. Contamination is a major issue, and the less chance of it, the better. When optimizing experiments with positive control samples, these should be performed in a different part of the lab, and if possible, with different equipment and reagents. Negative controls and aDNA experiments may be performed together in another section of the laboratory. The laboratory must be clean. Some aDNA labs even have air filtration systems to protect against contaminating DNA that is carried by the air. If possible, it may be a good idea to have a benchtop UV sterilizer of some sort, because it provides an enclosed environment within the lab, restricting airflow and providing UV lamps close at hand. One might also want to think about wearing sterile, protective clothing, if possible, and gloves at the very least. Note: gloves should be changed frequently.
As stated above, if possible, different equipment may be used for positive and negative controls. If this is not possible, that is probably ok. Also, any equipment used in aDNA research may not be used for any other research. Aerosol barrier tips for pipetting must be utilized at all times. Never use a tip more than once. All microfuge tubes and pipette tips should be autoclaved and UV sterilized. Also, pipettemen, gloves, scalpels, pestles, and any other equipment used in the research should be UV sterilized daily. A good idea is to just leave them under UV light after the experiments have been concluded for the day until you return.

All reagents, including alcohol (EtOH), MgCl₂, bleach, acid, DMSO, primers, buffers, dNTP's, oil, and most reagents in any kits used, should be UV sterilized before experiments start for the day for 20-30 minutes, except for any polymerases, proteinases, or DNA samples. Water used should be bought from a company, not taken from any tap, even taps that dispense double deionized water. Water should also be UV sterilized in its container. Any kits used for aDNA research should only be used for aDNA research and should be kept in the laboratory. UV sterilize any kit reagents that one thinks could be safely sterilized.

Reagents should be stored where indicated by the producer, but sterilized before being used for the day, except the reagents indicated above. For storage, positive control samples should never be stored in the same refrigerator or area where negative controls or ancient samples are being stored. In fact, positive samples should be in the laboratory as little as possible to reduce the risk of contamination. The storage unit should be as clean as possible, and cleaning and sterilizing it before starting research would be a very good idea.

**Optimizing Protocols:**

The first thing to do before starting any experiments on ancient samples is to optimize the protocols one will be utilizing to extract DNA from bones and to perform PCR upon. There really aren't many ways to optimize extraction protocols, but there are different things one can do with an extraction. These will be discussed later when protocols for the ancient samples are explained.
The one protocol that needs optimizing is PCR. PCR should be optimized with positive controls first, before any other aDNA research is done. After positive controls have been optimized, do not use them anymore in conjunction with ancient samples. When the actual ancient samples are being tested, there should always be at least one, usually two, negative controls. These are PCR reactions with more water added instead of sample DNA. In the case of nested PCR, where the product from the outer primer PCR is used to amplify with the inner primers, the negative controls should also be carried over to the next reaction. I will present two PCR protocols, both of which have worked for mitochondrial genomic DNA amplification. The first is one that we used for the majority of the aDNA research. It is a modified PCR protocol adapted for use with mitochondrial DNA for the amplification of 1-2kb fragments of DNA, which is a little much for aDNA research, but it worked very well. For both of these protocols, it is recommended that either Vent or Pfu DNA polymerase be used. With work such as this that is dependent upon exact sequence specificity, proofreading function provided by these two polymerases is necessary. Taq polymerase, the more widely used in PCR protocols, does not provide proofreading function. The use of DMSO, which functions to break up the secondary structure of DNA, making it more accessible, is optional, but it never hindered the reaction, so it wouldn’t hurt to put it in. Amplifications were performed in an AMPLITRON II (Thermolyne) thermocycler for 40 cycles of denaturation at 95 C for 50 seconds, annealing at 55 C for 50 seconds, and extension at 72 C for 2 minutes 30 seconds, with a 4 C chill upon completion.

Something to consider when optimizing with positive controls is the concentration of the positive samples. Too much DNA will hinder the PCR. A good concentration is somewhere near 50ng/μl, suspended in TE, though ancient extracts will most likely never reach this concentration.

The most important ingredient to optimize and the first factor that should be checked on is the MgCl₂ concentration. Magnesium, a divalent metal cation, is a necessary cofactor for many of the interactions used during PCR. PCR reactions will run best at certain concentrations of MgCl₂. It is usually best to start out, the first time PCR is run with positive controls, and run a range of MgCl₂ concentrations, from 1-5mM (ex: 5 reactions, the first with 1mM, the second with 2mM, etc.). Observe the resultant bands
on a gel and choose which concentration will work best for this set of experiments. One is urged to try both of the following two PCR protocols. The following is an example of a 40µl PCR reaction mix that we used many times to run PCR. 40µl is probably a good volume, as 100µl would be overkill and fairly dilute.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 10X</td>
<td>4.0 µl</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2.5 µl</td>
<td>50 mM</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.8 µl</td>
<td></td>
</tr>
<tr>
<td>The dNTP’s</td>
<td>3.2 µl</td>
<td>10 mM(2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 mM dTTP)</td>
</tr>
<tr>
<td>Primer 1</td>
<td>5.0 µl</td>
<td>4 pM/µl</td>
</tr>
<tr>
<td>Primer 2</td>
<td>5.0 µl</td>
<td>4 pM/µl</td>
</tr>
<tr>
<td>Polymerase</td>
<td>0.2 µl</td>
<td>5U/µl</td>
</tr>
<tr>
<td>Water</td>
<td>17.3 µl</td>
<td></td>
</tr>
<tr>
<td>Sample DNA</td>
<td>2.0 µl</td>
<td></td>
</tr>
<tr>
<td>PCR Oil</td>
<td>2 drops</td>
<td></td>
</tr>
</tbody>
</table>

Obviously, some of the ingredients (dNTP’s, Buffer, Primers, MgCl₂) will have to be adjusted depending on their stock concentrations. The PCR Buffer 10X used was from GibcoBRL. This protocol was prone to producing primer dimers, due to its high levels of primers and dNTPs present in the mix.

The following is a PCR protocol that was used near the end of the research with lower levels of many of the ingredients, also at a 40µl volume. Again, the Buffer 10X is from GibcoBRL.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 10X</td>
<td>4.0 µl</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0.7 µl</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2.5 µl</td>
<td>50 mM</td>
</tr>
<tr>
<td>The dNTP’s</td>
<td>1.0 µl</td>
<td>10 mM(2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 mM dTTP)</td>
</tr>
<tr>
<td>Primer 1</td>
<td>1.3 µl</td>
<td>4 pM/µl</td>
</tr>
<tr>
<td>Primer 2</td>
<td>1.3 µl</td>
<td>4 pM/µl</td>
</tr>
</tbody>
</table>
Polymerase  0.2μl  5U/μl
Water       27.0 μl
Sample DNA  2.0 μl
PCR Oil     2 drops

Again, please take into account stock concentrations.

Next, one would want to know just how sensitive PCR could be. Since aDNA research involves working with very small amounts of old, degraded DNA, one will want to check to see and optimize how little of samples one can amplify from. Run PCR on a positive control, find out how long the fragment being produced by the PCR is, clean up the reaction with a kit (to be discussed later), find the concentration, and serially dilute the product down to 100 templates. For calculation information, the New England Biolabs Catalog has conversion information regarding size and concentrations, as do other similar catalogs (The New England Biolabs Nucleic Acid Data page on page 284 of the 1998/1999 catalog has this information). From there, run PCR on the serial dilutions and see just how far down one can go in number of templates and still get amplifiable product. Svante Paabo, a master in the field of ancient DNA, was able to get amplification from 100 templates and up. Any lower than that yielded no amplification (Krings, 1997).

PCR products may be viewed on a 1%, 1.5%, or 2% agarose gel. The higher the percentage, the slower the DNA molecules will move through the matrix, but the separation between sizes will be better. Gels may be stained with an ethidium bromide solution so that DNA molecules may be visualized under UV trans-illumination.

Cleaning either extracted samples or PCR products will usually involve a kit. I recommend the QIAquick columns from QIAgen. They recover upwards of at least 85% of the DNA present. One can also use the BIO 101 GeneClean III kit, or any number of others. These kits rely upon separating DNA fragments larger than 40bp away from any other reagents present in a solution, and most are very good. The only problem lies in the fact that if something goes wrong, you won’t know what it is because the company won’t tell you what is in the kits. Thus, optimization is nearly impossible.

Sequencing is another reaction that will be a necessity for this research, though no one does their own sequencing anymore. Take your cleaned samples to whoever does the
sequencing on campus and get them sequenced. Make sure Vent or Pfu DNA polymerase is used. DNA sequencing used in this experiment involved producing single stranded molecules of the fragments amplified by PCR. This single stranded amplification was performed with the help of fluorescent dye-terminator ddNTPs. The PCR sequencing reaction ingredient list is listed below and adds to a total volume of 10μl.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminator Dye Premix</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Primer</td>
<td>0.5 μl 4pM/μl</td>
</tr>
<tr>
<td>Water</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>2.5X Sequence Buffer</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Purified Sample DNA</td>
<td>1.0 μl</td>
</tr>
</tbody>
</table>

The fluorescent dye terminator premix was from Perkin Elmer/ABI. The Sequence buffer contained MgCl₂. The PCR sequencing reactions were run for 25 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 15 seconds, and extension at 60°C for 4 minutes, with a 4°C chill at the end. Next, the products were cleaned and purified by running the samples through 400μl of Sephadex G-50 in Centri-Sep spin columns (Princeton Separations, Inc.) and vacuum dried. Samples were then resuspended in 1.0μl of loading buffer, which consisted of five parts deionized formamide to one part 30mg/ml blue dextran. Sequencing was then performed on an ABI 377 automated sequencer using 6% acrylamide gel.

There are many computer programs that help with comparing sequences, among them Sequencher™ 3.1 (Gene Codes Corp.). Also, keep in mind NCBI (the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) for sequence information and comparisons. To look for regions of similarity between sequences from different species, one may also use BlockMaker (http://blocks.fhcrc.org/), which will put together blocks of similar sequence based off sequences put in to the program. Primers may be constructed from these blocks.
Ancient Samples:

Ancient samples come in many shapes and sizes. Vertebrae are preferable, as they are thicker than other fish bones. The first thing to do is try to determine if the bones are salmonid. If you have salmonid specific primers, they won't work on other families of fish. Anthropologists may be able to tell the type of fish, if most of the skeleton was found. Other times, they may not, and one must take one’s chances. If possible, vertebrae with a diameter of at least 1.5cm, usually larger, are preferable.

Next, look at the state of the bones. If they look heavily degraded, they may not be worth experimenting upon. Try to determine where the bones came from and the type of climate in that area. Bones are well preserved in cold, arid climes, with little change in weather. Areas with changing seasons will not preserve bones as well, for the most part. Also determine the age of the bones. Anthropologists should have done this for you. Supposedly, DNA may be preserved in bones for up to 100,000 years, but fish bones may be a different story, as stated before. No one really knows how long fish bones will still yield amplifiable DNA.

A technique utilized by Svante Paabo called Amino Acid Racemization is used in many aDNA experiments (Poinar, 1996). It requires a portion of bone to be hydrolysed under acid conditions, and to have the subsequently released amino acids be analyzed under High Performance Liquid Chromatography and fluorescent detection. It requires around 10mg of bone, at least. The technique will probably have to be carried out in a biochemical lab with the proper equipment, etc. Based off the results from the amino acid racemization, one can sometimes tell whether the bones will be able to yield any amplifiable DNA. There is a correlation between the degradation of the bones and the amount of certain kinds of amino acids released after hydrolysis. One might want to read up on the procedure.

After one has determined to go ahead with the research, cleaning up and extracting from the bones is the next step. Most bone samples will be dirty and have many particles covering the outside. One may use water to clean off the bones, or tools to scrape off the outside particles. 10% Bleach has been tried in some experiments to help kill anything on the outside of the bones and to help in the cleaning. The bleach should only be applied for a few minutes, as you don’t want it to seep into the samples.
Also, rinse the samples at least twice with ddH2O after the bleaching. After cleaning, the bones should be crushed up in a microfuge tube using a pestle or other type of tool. The pestle worked ok, but it was still hard to get some big pieces really crushed well. From there, one can follow any basic DNA extraction protocol or kit. We used a solid tissue protocol PureGene® DNA Isolation Kit D-5000A from Gentra Systems, but there are many on the market.

After extractions, continue with PCR, though outer primers may not be successful, as ancient DNA is usually heavily fragmented, and there may only be fragments of up to 200bp present in the extract. One may have to amplify twice over with the inner primers. I encourage anyone to make their own primers if they feel that is necessary. Remember that primers should be anywhere from 20-30bp in length, have high G/C content, should not be complimentary to each other, and should have melting temperatures at or above 60 C. There are many computer programs to help with primer design, such as Amplify, etc. One may even want to design degenerate primers to make sure primers will bind to any possible change in sequence that may have occurred over time. Utilizing blocks of DNA sequence from related species constructed from BlockMaker, one can transfer those sequences to CODEHOP, accessible from the BlockMaker results page, and have CODEHOP construct degenerate primers for use in PCR. CODEHOP degenerate primers are better than normal degenerate primers, as they harbor two regions on the primers: one that is conserved and serves as a 5’ clamp, and a 3’ region that is variable and functions for the degenerate part of the primers. If using nested primers, keep in mind that they shouldn’t be too far apart, for reasons stated above.

I believe if one follows the above general outline for experiments on ancient fish research, a successful experiment could result. The preceding pages are a compilation of what I have learned during my stay in Dr. Thorgaard’s laboratory. Though it may not be apparent, I’ve learned much more about how research should be performed. Working in Dr. Thorgaard’s laboratory not only showed me what working in the lab is like, but also made me even surer that that is where I wanted to be.

I wish to thank everyone who has helped me in this endeavor. Thank you to Dr. Gary Thorgaard, who provided me the chance to work in his lab; to Kim Brown, who
provided friendship, guidance, and instruction; to Sarah Van Galder, who dug up the bones and ended up providing a newfound friendship; to Dr. Joe Brunelli, who provided insightful advice and commentary during the process; to Dr. Andrefsky, who authorized the use of the bones for this experiment; to Dr. Pat Carter, for the use of his lab room; and to everyone else in Dr. Thorgaard’s laboratory (Krista, Kyle, and Paul) who made me feel welcome during my stay. I appreciate it very much.

References:


Wisconsin Department of Natural Resources. Rainbow Trout (Oncorhynchus mykiss) illustration on front cover. Found at www.seagrant.wisc.edu/greatlakesfish/rainbowtrout.html

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