Kinship relations between the elite of Calakmul, México

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Research Year: 2006
Culture: Maya
Chronology: Early and Late Classic
Location: México
Site: Calakmul

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Abstract

This research funded by FAMSI is to establish methods to evaluate the DNA from a set of human skeletons pertaining to the Early and Late Classic royal tombs from Calakmul. This research is to analyze as many sets of remains as possible and to establish genetic relationships between the individuals.

SAMPLES:

The following samples were submitted for genetic analysis and research:

Ent 1*	Ent 18	II-5b '98
Ent 2*	Ent 19	II-6a '98
Ent 3	Ent 20	II-6b '98
Ent 4	Ent 21	IIA-1-99
Ent 6	Ent 22	E.II-c.1
Ent 7	Ent XV-2	IA-1-98
Ent 9*	Ent XV-3	IV-1

Ent 10	II-1 '97	IV-B-5
Ent 11	II-2 '97	IV-B-6
Ent 12	II-4a '97	IV-B-7
Ent 14	II-2 '97/98	IV-t.2 8B
Ent 15	II-4 '97/98	Utional Caan 1 '98
Ent 16	II-4b '97/98	Utional Caan 2 '98
Ent 17	II-5a '98	Kinich Pale #1

* Samples designated as priority samples for analysis.

METHODOLOGY:

All the procedures were performed in the clean laboratory of the Paleo-DNA Laboratory a highly specific laboratory designed for the analysis of ancient or degraded DNA. This laboratory has a separate sample storage location where the sample is received and stored in its receptacle. It has a dedicated clean laboratory area consisting of 4 rooms, one a reagent preparation room where no samples or modern DNA has ever entered. The second room is dedicated for the sample preparation, the third for experiment preparation and the fourth for access to each room through a hallway. The clean laboratory area has 2 independent air systems and is entered through an air shower. The individual working within this area must be dressed in a fully enclosed suit (part of face open) with hood, double booties, double sleeves, double gloves, face mask and eyewear. This clean laboratory is cleaned with bleach and sterilised every week including before and after workspace area use. All material within this laboratory are dedicated to this area and sterilised and cleaned within this area. No material that leaves the clean laboratory area is ever permitted back in due to the potential for contamination. The samples are passed through each room through dedicated through-the-wall pass-throughs. Amplification does not take place in the clean laboratory area that occurs in a dedicated room in the post analysis area. The post analysis area has an amplification room and a post amplification laboratory area. The work is conducted in a unidirectional fashion to eliminate the chance of cross-contamination between each area.

Decontamination

When first receiving a biological sample for DNA analysis, the handling of the sample prior to its arrival is not always known. Also, the environment in which the sample was originally discovered may contain substances that could inhibit the analysis. To prevent any contamination or inhibition, decontamination must be used. In this research the use of a 6% (w/v) sodium hypochlorite solution, and a 70% ethanol solution for decontamination was used this allows surfaces contaminants and exogenous DNA to be washed off. UV irradiation decontamination was also used by exposing the sample to

254 nm wavelength UV light source at a distance of 30 cm. This causes the formation of thymine dimers in any potential exogenous contaminating DNA.

Sample Preparation

In this project, a dremmel drill was used to prepare the biological sample for DNA extraction was used for these samples as it is advantageous since it collects a powdered sample from inside the biological source, therefore, collecting material that has not been exposed to the external environment or sources of contamination. If any particles were recovered throughout this process another method of pulverization using a mixer mill.

DNA Extraction

The method of DNA extraction that is used is dependent on factors such as the type and age of the sample, the taphonomic conditions in which the sample was found and how the sample is prepared prior to extraction. Two methods were used in this analysis, the enzymatic Proteinase K method and the chemical Guanidinium thiocyante method.

DNA Quantification:

In theory, there is a quantitative relationship that exists between the concentration of the template DNA before amplification and the final concentration of the amplified target DNA sequence at any cycle during PCR. Therefore, real-time PCR detects the increase in the amount of target DNA as the cycle number increases. There are different methods that can be used to detect the progress of the reaction. For this research two methods for detection were used; SYBR Green dye and the Applied Biosystems Quantifiler™ Human DNA Quantification Kit.

DNA Amplification

Following the extraction and purification of the DNA sample, a target region of interest on the DNA template is amplified to detect the presence of viable DNA and the degree of fragmentation of the template. This is referred to as detection PCR. All detection PCRs performed in this project was comprised of the following reagents and their corresponding final concentrations for each sample. The primers that are selected for the amplification of DNA are dependent upon the type of DNA being amplified and the target region of interest. In this research for mitochondrial DNA, only primers that have been previously developed and validated are used and target various fragment lengths of the mitochondrial D-Loop. Nuclear DNA primers were designed for a target region located on the Amelogenin gene on the sex chromosomes and kits for autosomal STRs were used for this analysis. Nested and hemi-nested PCR amplification is performed following the detection PCR. The PCR products from the detection PCR are purified using one of the commercial columns mentioned in the purification protocols sections. These purified products then undergo a second PCR with the same conditions as the detection PCR, except a different pairs of primers are used. For a nested PCR the primers selected anneal internally to the primers used in the initial detection PCR. In the case of a hemi-nested PCR, one of the primers used in the detection PCR is paired with a new primer that anneals internally to the other primer that was used in the detection PCR. For this research project, hemi-nested PCRs were conducted for mitochondrial DNA samples that were either highly degraded or considered ancient

samples. The nested PCR was performed on nuclear DNA for the amelogenin gene again for samples that were considered highly degraded or ancient.

Mitochondrial DNA Sequencing:

The mitochondrial DNA sequencing reaction that will be preformed during this project employs the chain terminating chemistry. More specifically, the ABI Big Dye® Terminator v3.1 Cycle Sequencing kit will be used to prepare the sequencing reactions, however the reagent volumes have been altered from those listed in the manufacturer's protocol.

Multiplex Autosomal Short Tandem Repeats:

Autosomal Short Tandem Repeats (STRs) will be amplified using a commercially available kit developed by Applied Biosystems. The AMPFl STR® Identifiler® PCR Amplification kit is able to amplify 15 loci from the autosomal chromosomes and the amelogenin locus on sex chromosomes simultaneously in one reaction. The kit uses 5-dye chemistry to allow differentiation of different groups of loci, when being run on an ABI PRISM® 3100 Genetic Analyzer.

DNA Amplification Detection:

Three methods were used for the analysis and detection of the amplified product. These three
techniques were agarose gel electrophoresis, ploy acrylamide gel electrophoresis and capillary
electrophoresis.

FINAL REPORT:

In the end the funding allowed for ten samples to be analysed. These included the designated priority samples.

Samples

Ent 1	Ent 14
Ent 2	Ent 15
Ent 3	Ent 20
Ent 9	Ent XV-2
Ent 12	Ent XV-3

These samples were extracted from between 095-0.171g of bone material. Every samples was extracted a minimum of two times each. The target amplification focused on the hypervariable region 1 and the 9bp deletion of the human mitochondrial genome. Each extract was amplified a minimum of four times for each amplicon. The most

successful amplification was obtained for at least the last half of the hypervariable region 1 from np16191 to np16420. Two profiles were generated for Ent. 2. At least one of these profiles has to be contamination. It is difficult to determine if the second profile is also contamination. From the data generated there is a close association between Ent. 9, Ent. 15 and Ent. XV-3. Also Ent. 2 shares many of the polymorphisms with these three individuals and could be closely related. Some of the samples did not produce a sequence different from the revised Cambridge reference Sequence (rCRS). It is possible that these samples could also be due to contamination as more genetic target regions within the mitochondrial genome would have to be tested to confirm.

Table 3. mtDNA sequence alignment of individuals

Anderson 16201	CAAGCAAGTA	CAGCAATCAA	CCCTCAACTA	TCACACATCA	ACTGCAACTC
Ent 1	....	....	....	....	....
Ent 2	....	....	..T.......	....	....
Ent 2	....	....	....	....	....
Ent 3	....	....	....	....	....
Ent 9	....	....	..T.......	....	....
Ent 12	....	....	....	....	....
Ent 14	....	....	....	....	....
Ent 15	....	....	..T.......	....	....
Ent 20	....	....	....	....	....
Ent XV-2	....	....	....	....	....
Ent XV-3	....	....	..T.......	....	....

Anderson 16251	CAAAGCCACC	CCTCACCCAC	TAGGATACCA	ACAAACCTAC	CCACCCTTAA
Ent 1	....	....	....	....	....
Ent 2	....	....	....	....	....
Ent 2	....	....	....	.........T	....
Ent 3	....	....	....	....	....
Ent 9	....	....	....	.........T	....
Ent 12	....	T.........	....	....	....
Ent 14	....	....	....	....	....
Ent 15	....	....	....	.........T	....
Ent 20	....	....	....	....	....
Ent XV-2	....	....	....	....	....
Ent XV-3	....	.........T	....	.........T	....

Anderson 16301	CAGTACATAG	TACATAAAGC	CATTTACCGT	ACATAGCACA	TTACAGTCAA
Ent 1	....	....	....	....	....
Ent 2	....	.........T	....	....	....
Ent 2	....	C.......A.	....	....	....
Ent 3	....	....	....	....	....
Ent 9	....	....	....	....	....
Ent 12	....	....	....	....	....
Ent 14	....	....	....	....	....
Ent 15	....	........A.	....	....	....
Ent 20	....	....	....	....	....
Ent XV-2	....	....	....	....	....

Ent XV-3	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .

Anderson 16351	ATCCCTTCTC	GTCCCCATGG	ATGACCCCCC	TCAGATAGGG	GTCCCTTGAC
Ent 1	. . . . .	.C.	. . . . .	. . . . .	. . . . .
Ent 2	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Ent 2	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Ent 3	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Ent 9	. . . . .	.C.	. . . . .	. . . . .	. . . . .
Ent 12	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Ent 14	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Ent 15	. . . . .	.C.	. . . . .	. . . . .	. . . . .
Ent 20	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Ent XV-2	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Ent XV-3	. . . . .	.C.	. . . . .	. . . . .	. . . . .

Anderson 16401	CACCATCCTC	CGTGAAATCA	ATATCCCGCA	CAAGAGTGCT	ACTCTCCTCG
Ent 1	. . . . .	. . . . .			
Ent 2	. . . . .	. . . . .			
Ent 2	. . . . .	. . . . .			
Ent 3	. . . . .	. . . . .			
Ent 9	. . . . .	. . . . .			
Ent 12	. . . . .	. . . . .			
Ent 14	. . . . .	. . . . .			
Ent 15	. . . . .	. . . . .			
Ent 20	. . . . .	. . . . .			
Ent XV-2	. . . . .	. . . . .			
Ent XV-3	. . . . .	. . . . .			

From the generated sequences it was possible to identify miscoding lesions. These miscoding lesions indicate the degree of degradation within the DNA. The degraded nature of the DNA was also assessed using multiplex PCR and it identified that 200bp or less was the average size of DNA fragments able to be recovered. The analysis has generated high costs due to the effort required to extract and analyse the degraded DNA. A novel method was established for the screening of samples to determine the quality of the recovered DNA. This screening method used gas chromatography mass spectroscopy to determine the degree of damage to the degraded DNA. This analysis supported the results generated by the miscoding lesions analysis.

CONCLUSION:

Genetic analysis was performed on ten individuals from Calakmul. From these ten individuals contamination was found in one sample and the possibility of maternal relationship in four other samples. However due to the high costs of the analysis of

highly degraded DNA samples further analysis on further individuals or more replicates and more genetic targets could not be performed. The damage to the DNA was identified through GCMS and miscoding lesion analysis.

PROTOCOLS:

Decontamination Protocol:

- Wipe the surface of the bone sample with a 6% (w/v) sodium hypochlorite solution.
- Rinse the surface of the bone sample with a 70% ethanol solution.
- Expose the sample to UV irradiation (254 nm wavelength) at a distance of 30 cm for over 24 hours.

Sample Preparation Protocol:

- Select a smooth surface free of any damage on the biological sample being prepared.
- Using a dremmel drill with a rotary disc to remove the surface of the bone.
- Using a dremmel drill with a regular bit, begin to drill on low speed. (It is important to drill slowly with pressure to prevent heating up of the drill or burning the sample)
- Collect the bone powder into a 1.5mL microcentrifuge tube.
- If any particles are recovered they are placed into the steel chamber with the steel ball of the mixer mill.
- Seal the chamber with tape and place the chamber horizontally into the mixer mill.
- Tighten the holder using the bolt at one end.
- Turn the mixer mill on for 20 seconds.
- Remove the chamber from the mixer mill and remove the seal.
- Aliquot the powder formed into 1.5mL microcentrifuge tubes.
- Store the sample dry, at room temperature until further analysis.

Proteinase K Extraction Protocol:

Proteinase K (PK) is an enzymatic extraction method that is usually used as the standard extraction method in forensic laboratories. The PK is used in conjunction with the denaturing ability of Sodium Dodecyl Sulfate (SDS) and Diothreitol (DTT) to create an extraction buffer to lyse the cells and release its cellular components, including DNA into solution. The enzymatic activity of PK is then able to denature proteins. The DNA is then isolated using one of the purification methods listed below. This extraction method will be used to extract DNA from various biological sources, including bone, teeth and tissue.

Required Reagents:
Proteinase K (20mg/mL)
TNE Buffer
20% SDS

0.39M DTT
ddH2O
Phenol
Chloroform: Isoamyl Alcohol (24:1 v/v)
3M Sodium Acetate
100% Ethanol
95% Ethanol
ddH2O

- Prepare 400µL 1X Extraction Buffer as follows; 290µL TNE Buffer, 40µL 20% SDS, 40µL 0.39M DTT, 2µL Proteinase K, and 28µL of water, and add to sample.
- Incubate at 37ºC in a fume hood overnight with gentle agitation.
- In a set of clean 1.5mL tubes, add 200µL of Phenol and 200µL of Chloroform: Isoamyl alcohol (24:1) to each tube.
- In a second set of clean 1.5mL tubes, add 400µL of Chloroform: Isoamyl alcohol (24:1) to each tube.
- Once the extraction solution completed its incubation period, 200µL of Phenol and 200µL of Chloroform: Isoamyl alcohol (24:1) was added directly to the extraction solution.
- The solutions were then centrifuged for 5 minutes at 13 000rpm.
- The aqueous layer was removed and placed in the previously prepared tube with 200µL of Phenol and 200µL of Chloroform: Isoamyl alcohol (24:1). It is important to note that none of the interphase containing denatured protein was removed.
- The solutions were vortexed and then centrifuged for 5 minutes at 13 000rpm.
- The aqueous layer was once again removed and placed into the previously prepared tube with 400µL of Chloroform: Isoamyl alcohol (24:1). This tube was vortexed and centrifuged for 5 minutes at 13 000rpm.
- The aqueous layer was removed and placed into a clean 1.5mL tube.
- If the sample was not subjected to an additional purification, the solutions were placed in a heating block at 65ºC with the caps open to allow any remaining chloroform to evaporate.
- Add 10% volume of 3M Sodium Acetate to the entire volume of the extract in clean sterile 2.0mL tube.
- Vortex the tube 1 minute.
- Add 2.5 times the volume of COLD 95% Ethanol to the tube containing the extraction solution.
- Place the tube on ice for 30 minutes.
- Centrifuge the tube for 5 minutes at 13 000rpm.
- Discard the supernatant liquid.
- Add 500µL ice cold 100% Ethanol to the tube.
- Vortex the tube for 1 minute, then centrifuge for 10 minutes at 13 000rpm.
- Discard the supernatant and let dry for 30 minutes.
- To resuspend add water and incubate at 37ºC for 15 minutes.

Guanidinium Thiocyanate Extraction Protocol:

This chemical extraction method is commonly used in research facilities. It uses the chaotropic agent, Guanidinium Thiocyanate (GuSCN) to dehydrate the available DNA and causing it to precipitate. From here, the DNA can be further purified using one of the purification methods described below. This extraction method will be conducted on modern, ancient and degraded biological sources, including bone, teeth and tissue.

Required Reagents:
4 M Guanidinium Thiocyanate (GuSCN) Extraction Buffer
Silica Beads Resin
Working Wash Buffer
100% Ethanol
ddH2O

- Add 500µL of 4M GuSCN (0.1M Tris-HCl pH 6.4, 0.02M EDTA pH 8.0, 1.3% Triton X-100) directly to the sample.
- Incubate at 56ºC overnight with gentle agitation.
- Boil the GuSCN extract at 94°C for 10 minutes and centrifuge at 13000 rpm for 1 minute.
- Remove the supernatant and place in a sterile 1.5 mL tube.
- Add 900 µL of GuSCN solution and 20 µL of silica to the sample and vortex briefly.
- Place the sample on ice for 60 minutes and invert the sample was inverted every 15 minutes to resuspend the silica.
- Spin the sample and discard the supernatant.
- Add 500 µL of Working Wash Buffer to the sample and vortex for 1 minute to resuspend the silica beads.
- Zip spin the samples, remove and discard the supernatant.
- Repeat the Working Wash Buffer wash step.
- Add 150 µL of 100% ethanol to the sample, vortex for 1 minute and then zip spin.
- Remove and discard the supernatant.
- Air dry the silica pellet for 30 minutes.
- Add 100 µL of ddH2O to the dry pellet and vortex to resuspend the DNA in the silica.
- Incubate at 56°C for 1 hour.
- Prior to the preparation of a PCR, centrifuge the sample at 13000rpm for 1 minute.

Bio-Rad Micro Bio-Spin® P-30 Microcentrifuge Chromatography Columns Protocol:

P-30 Microcentrifuge chromatography columns are used to separate macromolecules bas on the molecular weight of the components. The matrix within the column is comprised of porous polymeric beads. When the solution is added and centrifuged, the larger molecules will pass through the column, while the porous beads trap the smaller molecules. This results in a purified sample. These columns will be used as a additional purification step to remove any inhibitors that may have co-purified during the extraction and initial purification procedures.

Required Reagents:
Micro Bio-Spin® P30 Chromatography Columns

- Invert the column several times to resuspend the gel in the column and remove any bubbles.
- Snap the tip off and place column in a 2mL microcentrifuge tube and remove top cap.
- Centrifuge the column for 2 minutes at 3.4rpm to remove the packing buffer. Discard the buffer.
- Place the column into a clean 1.5mL tube. Apply thhe sample (20 – 75 µL volume) directly to the center of the column. It is important to not that by applying more or less than the recommended volume it may decrease the efficiency of the column.
- Centrifuge the column for 4 minutes at 3.4rpm. The purified DNA is now in solution with TRIS buffer.
- Discard the column.

SYBR Green DNA Quantification Protocol:

SYBR Green is a dye that binds to the minor groove of double stranded DNA. As it binds to the DNA, the intensity of its fluorescence increases. Therefore, as the amount of double stranded DNA increases, the intensity of fluorescence increases. This technique will be used to quantify bone, teeth and tissue samples.

For this technique, the same procedure for preparing a detection PCR is used but with the addition of 1.25 µL of SYBR Green dye.

Reagent	**Final Concentration**
10X Buffer, minus Mg	1X
50 mM MgCl2	200 µM
dNTP Mixture	0.2 µM
10 µM Forward Primer	0.2 µM
10 µM Reverse Primer	2 mM
Platinum Taq DNA Polymerase (5U/µL)	1.0 U
SYBR Green	
ddH2O	--*
Template	--*

*The volume of ddH2O added is dependent upon the volume of template added DNA that is added.

The cycling parameters for the SYBR Green Real-time PCR remained are as seen below. The number of cycles remained consistent regardless of the age and suspected degradation of the sample. Therefore allowing for relative quantification amongst different samples.

Temperature (°C)	**Time**	**Number of Cycles**
94.0	2:00	1
94.0	0:30	40
60.0*	1:00	
72.0	2:00	
4.0	Hold	1

*This annealing temperature may vary based on the sequence of the primer. The mitochondrial primers that are used in this project use the above annealing primer. However, for the nuclear primers the annealing temperature will be different and will be stated when discussing these procedures.

Applied Biosystems Quantifiler™ Human DNA Quantification Kit Protocol:

The Applied Biosystems Quantifiler™ Human DNA Quantification kit is a commercial kit that incorporates the 5'nuclease activity of AmpliTaq Gold® DNA Polymerase to cleave a TaqMan Probe that has annealed to the single stranded DNA downstream. When the probe is cleaved it will emit fluorescence. Therefore an increase in fluorescence indicates an increase in the number of single strands of DNA being amplified. This technique will be used to quantify extracted DNA samples from bone, teeth and tissues.

The reaction for each sample is prepared using the volumes listed in the table below.

Reagent	**Volume per Reaction (µL)**
Quantifiler™ PCR Reaction Mix	12.5
Quantifiler™ Human Primer Mix	10.5
DNA Template	2

Since this is a kit, specific cycling parameters have been developed to allow optimal amplification and the detection of fluorescence. These parameters are as follows;

Temperature (°C)	**Time**	**Number of Cycles**
95.0	10:00	1
95.0	0:15	40
60.0	1:00	
4.0	Hold	1

Detection PCR Protocol:

Reagent	**Final Concentration**
10X Buffer, minus Mg	1X
50 mM MgCl2	200 µM
dNTP Mixture	0.2 µM
10 µM Forward Primer	0.2 µM
10 µM Reverse Primer	2 mM
Platinum Taq DNA Polymerase (5U/µL)	1.0 U
ddH2O	--*
Template	--*

*The volume of ddH2O added is dependent upon the volume of template added DNA that is added.

Temperature (°C)	**Time**	**Number of Cycles**
94.0	2:00	1
94.0	1:00	30 – 45**
60.0*	1:00	
72.0	2:00	
4.0	Hold	1

*This annealing temperature may vary based on the sequence of the primer.

The mitochondrial primers that are used in this project use the above annealing primer. However, for the nuclear primers the annealing temperature will be different and will be stated when discussing these procedures.

**The number of cycles varies depending on the type and nature of the biological sample being tested. For modern samples, 30-35 cycles will be sufficient, where as 40-45 cycles will be used for ancient or degraded samples.

Mitochondrial DNA Amplification Primers:

Mitochondrial Primer and Sequence	Primer Length (bp)	Melting Temperature, Tm (°C)	GC Content (%)
mt15971F TTA ACT CCA CCA TTA GCA CC	20	58.35	45.0
mt16071F CCC ATC AAC AAC CGC TAT GT	20	60.4	50.0
mt16210F CCC ATG CCT ACA AGC AAG TA	20	56.0	45.0
mt16301F CAG TAC ATA GTA CAT AAA GCC A	22	57.8	36.4
mt1F GAT CAC AGG TCT ATC ACC C	19	60.5	52.6
mt15F CAC CCT ATT AAC CAC TCA CG	20	60.4	50.0
mt155F TAT TTA TCG CAC CTA CGT TC	20	56.3	40.0
mt247F GAA TGT CTG CAC AGC CAC	18	60.7	55.6
mt187R CGC CTG TAA TAT TGA ACG TAG G	22	61.5	45.5
mt279R GAT GTC TGT GTG GAA AGT GG	20	60.4	50.0
mt389R CTG GTT AGG CTG GTG TTA GG	20	62.5	55.0
mt429R CTG TTA AAA GTG CAT ACC GCC A	22	61.5	45.5
mt16114R mt16257R mt16322R TGG CTT TAT GTA CTA TGT ACT G	22	57.8	36.4
mt16420R TGA TTT CAC GGA GGA TGG TG	20	60.4	50.0

Mitochondrial DNA Sequencing:

The mitochondrial DNA sequencing reaction that will be preformed during this project employs the chain terminating chemistry. More specifically, the ABI Big Dye® Terminator v3.1 Cycle Sequencing kit will be used to prepare the sequencing reactions, however the reagent volumes have been altered from those listed in the manufacturer's protocol.

Required Reagents
Big Dye® Terminator v3.1

- For each DNA sequencing reactions, add the following reagents into a 0.2mL tube; 3.0 µL of Big Dye® Terminator v3.1, 0.3 µL of either the forward or reverse primer used in the detection PCR and 7.0 µL of the purified PCR product.
- The DNA sequencing reaction is then amplified using the following PCR cycling parameters.

Temperature (ºC)	**Time (minutes)**	**Number of Cycles**
94.0	0:30	35*
50.0	0:15	
72.0	4:00	
4.0	Hold	1

*Please Note that for ancient or degraded samples the cycle number was sometimes increased to 40 cycles.

- Purify the sequencing PCR product using either the Dyex or E.Z.N.A size exclusion DTR columns mentioned in the purification protocols section.
- Desiccate the purified sequencing product.
- Store the sample at 4 ºC until sequencing detection with the ABI PRISM 3100 Genetic Analyzer.

Nuclear DNA Amplification Primers

Primer Primer and Sequence	Length (bp)	Melting Temperature, Tm (°C)	GC Content (%)
Amel-A CCC TGG GCT CTG TAA AGA ATA GTG	24	65.4	50.0
Amel-B ATC AGA GCT TAA ACT GGG AAG CTG	24	63.7	45.8
AmF GCT ACC ACC TCA TCC TGG GCA CCC	24	64.0	67.0
AmR ACA GGC TTG AGG CCA ACC ATC AGA GC	26	63.0	58.0

Multiplex Autosomal Short Tandem Repeats:

Autosomal Short Tandem Repeats (STRs) will be amplified using a commercially available kit developed by Applied Biosystems. The AMPFl STR® Identifiler® PCR Amplification kit is able to amplify 15 loci from the autosomal chromosomes and the amelogenin locus on sex chromosomes simultaneously in one reaction. Since it is a commercial kit, the reagents used in the reaction are proprietary. However, it is known that the kit uses 5-dye chemistry to allow differentiation of different groups of loci, when being run on an ABI PRISM® 3100 Genetic Analyzer.

Required Reagents
AmpFl STR Identifiler Mastermix
AmpFl STR Identifiler Primer Mix
AmpliTaq Gold Polymerase (5U/µL)
ddH2O

- For each AmpFl STR® Identifiler® PCR amplification sample, the following reagents are combined in a 0.2mL tube.

Reagent	**Volume Per Reaction (µL)**
AmpFl STR Identifiler Mastermix	10.5
AmpFl STR Identifiler Primer Mix	5.5
AmpliTaq Gold Polymerase (5U/µL)	0.5
ddH2O	--*
Template DNA	--*

*The total volume of the reaction is 25µL. However, only 15 µL of the mastermix, primer and AmpliTaq cocktail will be added to the reaction, while the extra volume is allowed for pipetting error. The volume of ddH2O added is dependent on the volume of template added.

The AmpFl STR® Identifiler® reaction mix is amplified using the following PCR cycling parameters.
Following the amplification, cover the products in aluminum foil. Due to the fluorescently labeled primers the reactions are light sensitive.
Store the samples at 4 ºC until fragment analysis is conducted on the ABI PRISM® 3100 Genetic Analyzer.

Temperature (ºC)	**Time (minutes)**	**Number of Cycles**
95.0	11:00	1
94.0	1:00	28
59.0	1:00	
72.0	1:00	
60.0	1:00:00	1
4.0	Hold	1

Agarose Gel Electrophoresis Protocol:

Agarose gel electrophoresis is a detection method that will be used to detect the absence or presence of DNA, as well as for the comparison of different sizes of amplified DNA fragments throughout this project. Agarose gels however, have a lower resolution than some of the other detection techniques and therefore are not useful for differentiating fragments that differ by only a few base pairs in length.

Required Reagents
1X TBE Buffer
Agarose
10mg/mL Ethidium Bromide

Gel Making Protocol

- *The following procedure will make a 1-1.5% agarose gel.
- Weigh 0.375g of agarose and pour into a 125 mL Pyrex flask.
- Measure 25mL of 1X TBE Buffer and slowly add it to the agarose, while swirling the flask continuously.
- Cover the top of the flask with aluminum foil.
- Heat the solution while swirling every 1-2 minutes until agarose is completely dissolved.
- Allow the solution to cool for 5 minutes.
- Add 2.0 µL of 10mg/mL ethidium bromide stain.
- Slowly pour solution into an agarose plate and insert the gel comb.
- Allow the gel to settle for 30-45 minutes at room temperature. The gel may also be placed in the fridge for less time.

Loading and Running the Gel

- Once the gel has settled, slowly remove the comb from the gel and carefully place the gel into the gel apparatus.
- Fill the gel apparatus with 1X TBE Buffer until the gel is completely submerged. There is usually a fill line indicator on the side of the apparatus.
- Purge the wells in the gel with a Pasteur pipette to ensure no air bubbles are present.
- Mix 7.0 µL of the PCR product with 3.0 µL of 6X Loading Dye and a sample to each well of the gel (Be sure to leave one well empty)
- Load 3.0 µL of a DNA size standard in the one remaining well.
- Set the voltage to 110 volts and the time for 25 minutes on the electrophoretic power pack, and plug the gel apparatus into the power pack.
- Once the gel has run, place the gel on the transilluminator (set at wavelength UV B) and photograph.

Polyacrylamide Gel Electrophoresis (PAGE) Protocol:

Polyacrylamide (PAGE) gels are the second technique that will be used for detection of amplified DNA during this research project. This detection technique is advantageous as it is highly sensitive to low concentrations of PCR products. As well, it is capable of resolving fragments of DNA that differ only by one base pair in length. PAGE gels will be used for analysis of the amelogenin gene, nested and hemi-nested PCRs and for the comparison of different size fragments.

Required Reagents
1X TBE Buffer
5X TBE Buffer
Acrylamide
TEMED
10% APS
ddH2O

Gel Making Protocol

- *This protocol will result in 12 – 6% PAGE gels
- The 10% APS solution is prepared by mixing 0.1 g APS to 1.0 mL ddH2O.
- Chill a 125 mL Pyrex flask on ice.
- Set out 12 gel cassettes and combs prior to making the solution.
- Combine 20 mL 5X TBE Buffer, 12.5 mL Acrylamide, 66.5 mL ddH2O, 900 µL of 10% APS, and 90.0 µL of TEMED into the Pyrex flask.
- After mixing, keep the solution on ice and continue to swirl every 2 minutes.
- Fill the gel cassettes with the solution using a transfer pipette. It is important to tilt the cassette and fill one corner at a time to avoid the formation of bubbles.
- Place the comb into the top of the filled cassette.
- Allow the gel to settle standing up for 1 hour at room temperature.
- Store the gels at 4 ºC until ready to use.

Loading and Running the Gel
Remove the white tape on the bottom of the gel and the gel comb from the gel cartidge.
Slide the gel cartridge into the gel apparatus. Each apparatus may hold up to two gels, if only 1 gel is being loaded place an empty cassette or a spacer in the other slot.
Fill the gel apparatus with 1X TBE Buffer until the wells of the gel are completely submerged in the centre of the apparatus and the slot at the bottom of the gel cassette is also submerged.
Purge the wells with a Pasteur pipette ensuring no air bubbles are present.
Combine 7 µL of the PCR product with 3 µL of 6X Loading Dye.
Load 3 µL of the DNA size standard into 1 well, followed by the samples into the remaining wells.
Connect the gel apparatus to the electrophoretic power pack and adjust the voltage to 125 volts and the time to 40 minutes.
Once the run is completed, open the gel cassette and carefully remove the PAGE gel.

Stain the PAGE gel for 15 minutes with Ethidium Bromide.
Place the gel on the transilluminator (set at wavelength UV B) and photograph.

Capillary Electrophoresis Protocol:

The detection of autosomal STRs and mitochondrial DNA sequencing will be achieved using an ABI PRISM® 3100 Genetic Analyzer. This technique has the ability to not only to differentiate DNA fragments by a single base pair, but can also analyze multiple fragments of the same length with the use of fluorescently labeled primers during amplification.

Genescan Fragment Analysis Detection
Required Reagents
Hi–Di Formamide
Size Standard (usually 500-LIZ)

Preparation and Loading of Sample

- Resuspend the desiccated sample to be sequenced in 15 µL of Hi-Di Formamide.
- Vortex the sample for 1 minute and zip spin the tube.
- Heat the sample at 95 ºC for 3 minutes and then immediately place on ice for 2 minutes.
- Vortex and zip spin the sample.
- Hold the sample on ice until ready to load for capillary electrophoresis.
- Transfer the entire 15µL volume sample into a 96 well ABI Plate.
- Be sure that the 16 wells that correspond to a capillary during analysis contain either 15µL of sample or Hi-Di Formamide.
- Centrifuge the ABI Plate to remove any air bubbles prior to loading the plate in the ABI PRISM® 3100 Genetic Analyzer.

Fragmentation Analysis using the ABI AMPFISTR® Identifiler Kit Protocol:

Required Reagents

Hi-Di Formamide
500-LIZ Size Standard

Preparation and Loading of Sample

- Label a 0.5 mL tube for each sample, and an additional tube for the Allelic Ladder sample.
- Add 10 µL of the sample to the appropriately labeled tube and 10 µL of the Allelic Ladder to its labeled tube.
- Add 0.3 µL of the 500-LIZ Size Standard and 9.0 µL of Hi-Di Formamide to each tube.
- Vortex the sample and then zip spin.
- Heat the sample to 95 ºC for 3 minutes, and then immediately place on ice for 2 minutes.
- Briefly vortex and zip spin the sample.
- Hold the sample on ice until ready to load for capillary electrophoresis.
- Transfer the entire volume of the sample (10.3 µL) into a well on a 96 well ABI Plate.
- Be sure that the 16 wells that correspond to a capillary during analysis contain either 15µL of sample or Hi-Di Formamide.
- 10. Centrifuge the ABI Plate to remove any air bubbles prior to loading the plate in the ABI PRISM® 3100 Genetic Analyzer.