Research Article

Advances in Bioengineering & Biomedical Science Research

The Discovery of the "Skipping Generations" Phenomenon

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Abstract

Statement of the Problem: *The demanding need to discover someone's identity is not possible with the nuclear DNA especially when the traces are highly degraded, since the nuclear DNA is destroyed in these conditions. Only the mitochondrial DNA that is inherited maternally can survive in these compromised conditions. The purpose of this study is to find a genetic commonality between UAE nationals.*

Methodology and Theoretical Orientation: *150 buccal swabs of unrelated UAE female students (approved by the UAE ID) of Sharjah Higher Colleges of Technology were collected and kept at room temperature for a period of three months or longer; to destroy the nuclear DNA, so only the mtDNA is present. mtDNA testing was performed on these buccal swabs, and it's consisting of DNA Extraction, Real-Time quantitative PCR, Cycle sequencing and Capillary electrophoresis. The ABI PRISM®310 Genetic Analyzer capillary autosequencer [ABI PRISM® SeqScape® Soft-ware Version 2.6] was used to generate the mitochondrial DNA profiles.*

Findings: *From these haplotype data, a total of 229 polymorphisms were observed carefully. 106 different polymorphisms were identified out of them, and classified into unique and common polymorphisms. Interestingly, two individuals from the study subjects lacked unique polymorphisms.*

Conclusion and Significance: *It's impossible for anyone to preserve their mtDNA from their great ancestors till now. The discovery of the remains of the Romanov family back in 1991 concluded that the comparison of mtDNA that is more than three generations old is more likely to get at least one mutation in the current generation. Therefore, if a vertical study is done on those two individuals with their older generations, definitely they will have unique polymorphisms compared to their older generations. Those two individuals are the effect of "Skipping Generations" phenomenon, the term that I have invented to solve the mystery of having two individuals with no unique polymorphisms.*

Keywords: Haplotype, Mitochondrial DNA, Polymorphisms, "Skipping Generation" phenomenon

Introduction

The mitochondrion is a two-walled organelle that is present within the cytoplasm of a human cell. Tao reports, in the 1840s, Carl Benda is the one who discovered the mitochondria, when he was examining a living cell by a light microscope, and named them as such so [1]. The mitochondrion is like a factory that produces cellular energy. Tao adds that in the 1930s, Hans Krebs is the one who stated the function of the mitochondria through a process called oxidative phosphorylation, which is a process that produces ATPs that can transport chemical energy that fuels the entire cell [1]. The mitochondrion has its own DNA that is different from the nuclear DNA, as described in Figure 1.Tao writes, Margit Nass and Sylvan Nass were the ones who noted the presence of small circular double-stranded DNAs within the mitochondria in the 1960s [1]. The mitochondrial DNA has two regions, which are the coding and non-coding regions. Evans observes that the coding region of the mitochondrial DNA has 37 transcribed genes that code for products that are used in the oxidative phosphorylation process and these are 13 proteins, 2 ribosomal RNAs and 22 transfer RNAs [2]. Butler points out, the non-coding region of the mitochondrial DNA that doesn't code for any gene products is called as the control region, which has Hypervariable Region 1 and Hypervariable Region 2 that most of the forensic DNA studies are focused on [3]. The mitochondrial DNA is strictly inherited from the mother. Reid describes that during spermatogenesis, the sperm mitochondria are tagged with ubiquitin, which is a protein that signals for degradation, as a consequence, if any sperm mitochondria entered a fertilized egg, they will be selectively destroyed, and that explains the maternal inheritance of the mitochondrial DNA [4]. The mitochondrial DNA can survive in highly degraded biological samples. Butler contends, heat and humidity has no effect on getting the mitochondrial DNA to be

damaged due to two main reasons; first, a single cell can accommodate hundreds of mitochondria and each mitochondrion contains 5 copies of the mitochondrial DNA, second, exonucleases cannot break the mitochondrial DNA because of its circular nature, these two reasons increase the rate of survival in highly degraded biological samples compared to the nuclear DNA [3]. Mitochondrial DNA testing takes advantage of the mitochondrial DNA's exceptional characteristics; to successfully identify unknown compromised biological remains. All mitochondrial DNA profiles are compared to the Anderson sequence, and any base change in the mitochondrial DNA profile that is different from the Anderson sequence is considered as a polymorphism. Butler emphasizes that in 1981, Anderson is the one who first sequenced the whole mitochondrial genome, and the Anderson sequence is called now a days as the revised Cambridge Reference Sequence, which is all the mitochondrial DNA profiles are compared to [3]. The mitochondrial DNA has a high mutation rate due to having few DNA repair mechanisms. Butler confirms, because of the high mutation rate of the mitochondrial DNA, there is an increased variability between individuals, so they can be classified into haplogroups according to their common polymorphisms [3]. The aim of the work undertaken is to find a similarity between the UAE nationals that is unique enough to recognize them as a haplogroup. But through looking at the differences, we have discovered the "Skipping Generations" phenomenon.

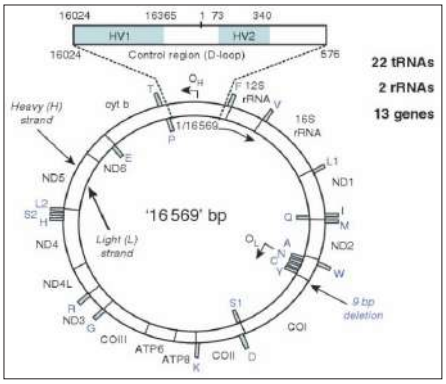


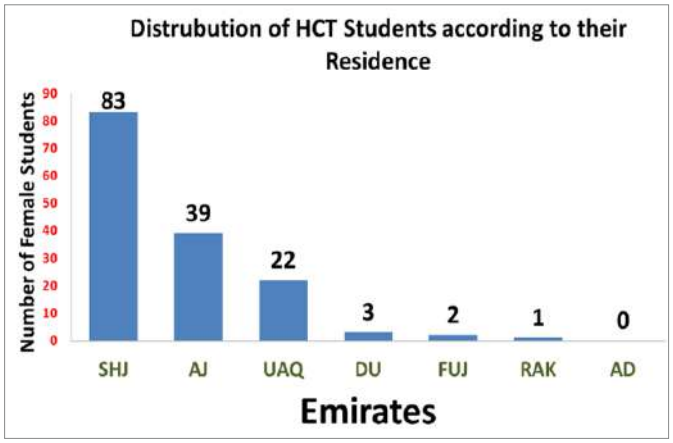
Figure 1: The structure of the mitochondrial DNA. It is a circular genome that has a heavy strand from the outside and a light strand from the inside, it is about 16569 base pair long, and it has a coding region and non-coding region, and the non-coding region is called the control region that contains Hypervariable Region 1 (HV1) and Hypervariable Region 2 (HV2), which are most targeted by the forensics [5]

Materials and Methodology

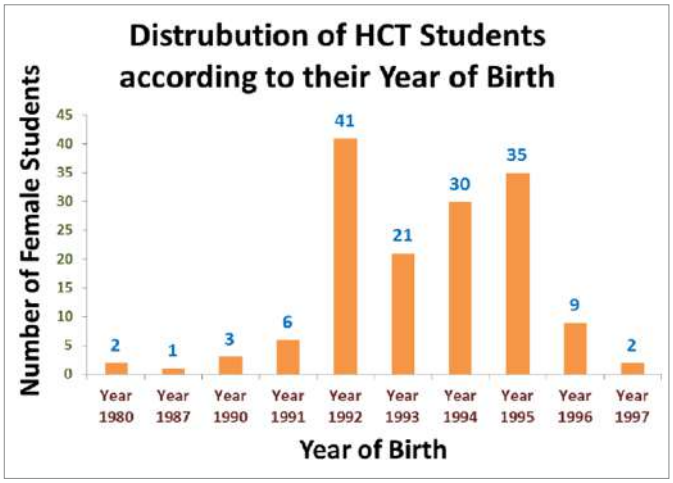
Data Collection

Buccal swabs of 150 unrelated UAE female students (approved by the Emirates identity card) of Sharjah Higher Colleges of Technology were collected from each individual's cheek in a period from 26/10/2014 to 16/10/2014. Some of those students were from Sharjah, Ajman, Umm Al Quwain, Dubai, Ras Al Khaimah and Fujairah (Appendix D). 148 of them were 18 year old to 28 year old, whereas the other two were 35 year old (Appendix E). 67 different surnames were identified and 7 students don't have any family name, as shown in Figure 2. The inclusion and exclusion criteria are listed in Table 1, since only 86 of the 150 students were accepted to be enrolled in our study. The sample size estimation of our research is based on the Confidence interval-based formula, and according to this formula, we are targeting only 11% of the whole UAE population, as explained in Figure 3. All the buccal swabs were air-dried at room temperature in a safety cabinet overnight. Then, in the following morning, all the samples were tightly closed and kept on the bench at room temperature for a period of three months or longer; to destroy the nuclear DNA. Therefore, the only DNA that will be present in those samples is the mitochondrial DNA.

Appendix D



Appendix E



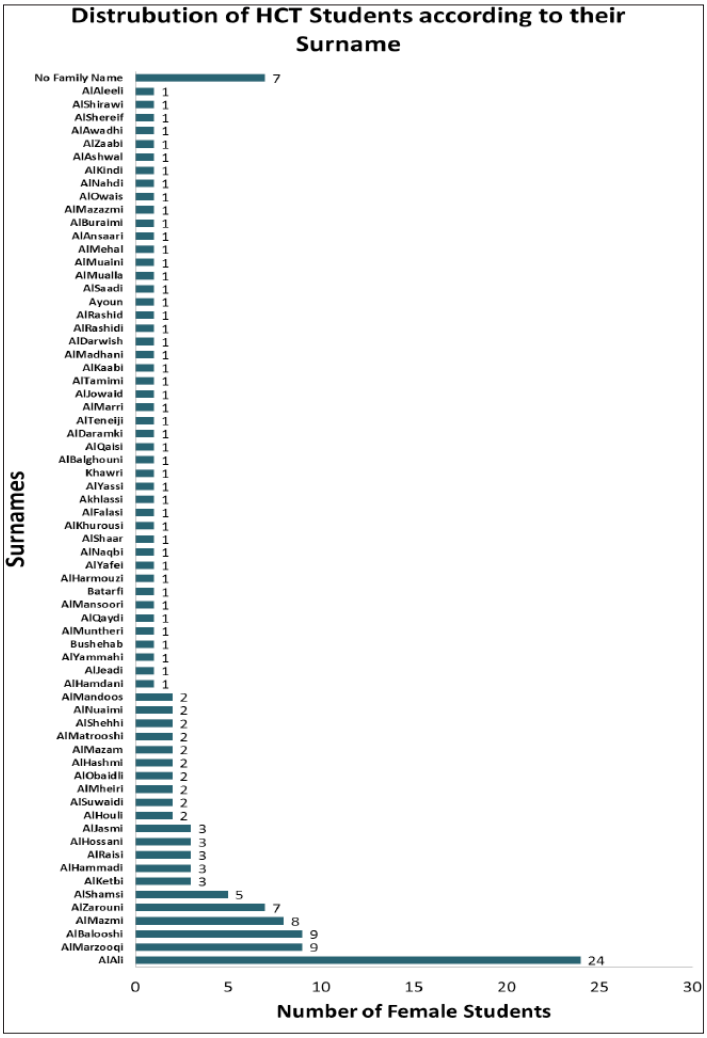


Figure 2: List of all the different family names that were identified: The largest group was from AlAli

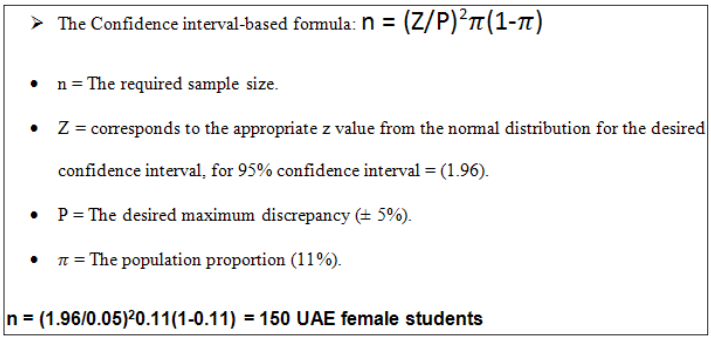


Figure 3: Sample Size Estimation: The number of UAE female students that should be enrolled in our study is based on the Confidence interval-based formula

Table 1: The Inclusion and Exclusion Criteria

Inclusion Criteria	Exclusion Criteria
1. Age between 18 years old to 35 years old. Children won't understand the purpose of this research and they won't give useful or correct information about the origin of their family. However, elderly people won't cooperate with us; they won't tell us about their real age as they are going to pretend that they are from a younger generation, and this is may affect the whole outcome of the research. 2. Female student from Sharjah Higher Colleges of Technology (Sharjah Women's College). We have the access to the student schedule for any female student from Sharjah Women's College only. Therefore, female students will be available in the college according to their schedule, so if there is any further investigation, we know how to reach them. Male students who are from Sharjah Men's College are not part of our study due to the lack of availability, since we don't have the access to their student schedule.	1. Female faculty from Sharjah Higher Colleges of Technology (Sharjah Women's College). In our study, we are required to get some personal information, as a rule we are not allowed to get any personal information from any teacher in our college. 2. Individuals who are maternally related. For example, sisters are maternally related; they have the same mitochondrial DNA, which was inherited from their mother. As a result, the outcome of the study will be bias toward one family name than the other.
	3. Refuse to give informed consent. Any individual who don't want to be informed about the purpose of the study, the possible outcomes of the study and the meanings of the outcomes is immediately excluded from the study, because that individual won't cooperate with us if there is any further investigation.

DNA Extraction

Randomly, out of the 86 buccal swabs, 50 buccal swabs were extracted along with extraction negative controls. The extraction process is done by the AutoMate Express™ Instrument by using the PrepFiler Express™ Forensic DNA Extraction kit (Appendix F). The addition of proteinase K is not included in the Our DNA extraction protocol, because our AutoMate Express™ Instrument works by the Magnetic Beads technology. Proteinase K is an enzyme that digests proteins, which is used to remove contaminating proteins that are present in the sample that needs to be purified, or to destroy a cellular matrix that is made of proteins, such as the structure of the bone. In our case, we don't have cellular matrixes that are made of proteins, since they are only buccal cells. However, contaminating proteins are major risk that threatens our specimens, and the Magnetic Beads technology eliminates this risk without the use of proteinase K. The concept of the Magnetic Beads technology relies on the attraction of the apposite charges and the repulsion of the same charges. The DNA is always negatively charged, while the protein can be negatively or

positively charged depends on the pH environment of the aqueous wash buffer (whether it is low or high pH). Therefore, the DNA that is negatively charged will attract to the magnetic bead that is positively charged, and the pH environment of the aqueous wash buffer will be low, so the protein will be positively charged, as a consequence, the protein will repel from the magnetic bead. As a result, the DNA purification will be free from any contaminating proteins.

Appendix F

DNA Extraction Protocol

DNA Extraction

1. Change the previous laboratory coat and wear a new one just for the Pre-PCR section (Name it as Pre-PCR laboratory coat).
2. Wear a mask and double gloves.
3. Disinfect the inner part of the Safety cabinet with 70% Isopropyl Alcohol wipes.
4. Disinfect the 300µL pipette, the two racks, the outside of the tips boxes and the marker pen with 70% Isopropyl Alcohol wipes.
5. Remove the double gloves, and wear new double gloves. Then, wash the double gloved hands with 95% Ethanol solution.
6. Use the marker pen to label the Filter tubes (on the top and the side of the tubes) and the Elution tubes (on the top of the tubes only) to their corresponding code number.
7. Choose an area in the Safety cabinet where the buccal swabs can be cut (Name it as the Cutting area).
8. Disinfect the Cutting area with 5% Sodium hypochlorite solution, and followed by with 95% Ethanol solution.
9. Place a filter paper on the Cutting area.
10. Take out the desired buccal swab from its transport tube and place it vertically on the center of the filter paper (the cotton area of the buccal swab is faced down).
11. Use the other hand to grape a sterile surgical blade to cut one fourth of the cotton area of the buccal swab (Name it as the desired portion).
12. Put the buccal swab into its transport tube.
13. Use the same surgical blade to catch the desired portion and place it into its corresponding Filter tube.
14. Place the surgical blade that has been used in the center of the filter paper; to cover it with the filter paper in order to discard it into its appropriate discarding bin.
15. Wash the double gloved hands with 95% Ethanol solution.
16. Disinfect the Cutting area with 5% Sodium hypochlorite solution, and followed by with 95% Ethanol solution.
17. Repeat step 9, 10, 11, 12, 13, 14, 15 and 16 for the next buccal swab.
18. Dispense 300µL of the Lysis Buffer solution into each Filter tube.
19. Place the Filter tubes in the ThermoMixer at 70OC for 20 minutes.
20. At the meantime, prepare the AutoMate Express™ Instrument by disinfecting the Cartridge rack and the Tip&Tube rack with 70% Isopropyl Alcohol wipes.
21. Take out one reagent Cartridge for each sample tube, and shake them for 15 seconds.
22. Load the desired number of reagent Cartridges in the Cartridge rack.
23. Insert the loaded Cartridge rack into the AutoMate Express™ Instrument.
24. Place the Elution tubes in the E-row of the Tip&Tube rack, and open them.
25. On the same rack, place the Tips that are inserted into the Tip Holders in the front of each Elution tube in T1-row.
26. After the ThermoMixer is finished, centrifuge the Filter tubes for 3 minutes at 400 rpm [Revolutions Per Minute].
27. After the centrifugation is finished, take out the filter part of each the Filter tube, and discard it into its appropriate discarding bin.
28. On the Tip&Tube rack, place the other parts of the Filter tubes in front of their corresponding Elution tube in S-row.
29. Insert the loaded Tip&Tube rack into the AutoMate Express™ Instrument.
30. Set up the right protocol for the AutoMate Express™ Instrument by pressing ENTER, No.1, ENTER. Then, start the process by pressing START. The DNA Extraction process will take 30 minutes.
31. After the DNA Extraction process is finished, place the Elution tubes in the refrigerator at 4°C until the next step.

Mito-PCR

Out of the 50 samples that have been extracted, only 21 samples were selected to be proceeding to the rest of the Mitochondrial DNA test. We have used the AmpliTaq Gold® 360 Master Mix kit from the Applied Biosystems® to prepare a polymerase chain reaction (PCR) for those 21 samples (Appendix G). We have targeted Hypervariable Region 1 and Hypervariable Region 2 of the mitochondrial genome to perform our research on (Appendix H). The design of the forward primers and reverse primers for Hypervariable Region 1 and Hypervariable Region 2 are listed in Table 2. We have used the MultiGene Thermal Cycler to do our PCR reaction on, which is mainly consist of 34-38 cycles of denaturation at 94°C; to separate the double-stranded DNA, primer annealing at 56°C; to let the primers attach to the DNA, and extension by DNA polymerase at 72°C; to let the DNA to be synthesized. This technique is used to double the amount of the DNA.

Appendix G

Mito-PCR Protocol

1. Wear the Pre-PCR laboratory coat.
2. Wear a mask and double gloves.
3. Disinfect the inner part of the PCR WorkStation with 70% Isopropyl Alcohol wipes.
4. Disinfect the pipettes, the PCR racks, the outside of the tips boxes and the marker pen with 70% Isopropyl Alcohol wipes.
5. Remove the double gloves, and wear new double gloves. Then, wash the double gloved hands with 95% Ethanol solution.
6. Use the marker pen to label the PCR tube (on the top and the side of the tube) as HV1 with the sample code number. Take another PCR tube and label it as HV2 with the same sample code number. So, each sample must have two PCR tubes, one for HV1 and the other one for HV2.
7. Repeat step 6 for the next sample.
8. Dispense 12.5 µL of AmpliTaq Gold® 360 Master Mix, 1.25 µL of Forward Primer [HV1], 1.25 µL of Reverse Primer [HV1] and 9 µL of Nuclease-Free Water into each (HV1) PCR tube.
9. Dispense 12.5 µL of AmpliTaq Gold® 360 Master Mix, 1.25 µL of Forward Primer [HV2], 1.25 µL of Reverse Primer [HV2] and 9 µL of Nuclease-Free Water into each (HV2) PCR tube.
10. Take 1 µL of each extracted DNA, and put them into their corresponding PCR tubes [according to their sample code numbers].
11. Centrifuge the (HV1) and (HV2) PCR tubes for 1 minute at 14.1 rcf [Relative Centrifugal Force].
12. Remove the Pre-PCR laboratory coat (to enter the Post-PCR

section) and wear a new one just for the Post-PCR section (Name it as Post-PCR laboratory coat).

13. Put the (HV1) and (HV2) PCR tubes in the MultiGene Thermal Cycler. Run the "Mito-PCR" protocol, after typing the total volume = 25 µL. The "Mito-PCR" process will take 2 hours and 41 minutes.
14. After the "Mito-PCR" process is finished, place the (HV1) and (HV2) PCR tubes in the refrigerator at 4°C until the next step.

Appendix H

The selected regions of the Mitochondrial DNA from Anderson sequence

Hypervariable Region 2 (15-484)

1	gatcacaggtctatc**accctattaaccactcacgggagctctccatgcatttggtatttt**
61	**cgtctgggggtgtgcacgcgatagcattgcgagacgctggagccggagcaccctatgtc**
121	**gcagtatctgtctttgattcctgcctcattctattatttatcgcacctacgttcaatatt**
181	**acaggcgaacatacctactaaagtgtgttaattaattaatgcttgtaggacataataata**
241	**acaattgaatgtctgcacagccgctttccacacagacatcataacaaaaaatttccacca**
301	**aaccccccctccccccgcttctggccacagcacttaaacacatctctgccaaaccccaa**
361	**aaacaaagaaccctaacaccagcctaaccagatttcaaattttatctttaggcggtatgc**
421	**acttttaacagtcacccccaactaacacattatttttcccctcccactcccatactacta**
481	**atct**catcaatacaacccccgcccatcctacccagcacacacacaccgctgctaacccca

Hypervariable Region 1 (15971-16451)

15961	agaaaaagtc**tttaactccaccattagcacccaaagctaagattctaatttaaactattc**
16021	**tctgttctttcatggggaagcagatttgggtaccacccaagtattgactcacccatcaac**
16081	**aaccgctatgtatttcgtacattactgccagccaccatgaatattgtacggtaccataaa**
16141	**tacttgaccacctgtagtacataaaaacccaacccacatcaaaccccccccccccatgct**
16201	**tacaagcaagtacagcaatcaaccttcaactatcacacatcaactgcaactccaaagcca**
16261	**cccctcacccactaggataccaacaaacctacccacccttaacagtacatagtacataaa**
16321	**gtcatttaccgtacatagcacattacagtcaaatcccttctcgtccccatggatgacccc**
16381	**cctcagataggggtcccttgaccaccatcctccgtgaaatcaatatcccgcacaagagtg**
16441	**ctactctcctc**gctccgggcccataacacttgggggtagctaaagtgaactgtatccgac

Table 2: Primer Sequences for PCR and Sequencing Reactions for Hypervariable Region 1 and Hypervariable Region 2 of the Mitochondrial Genome

Region of the Mitochondrial Genome	Position	Primer Type	Primer Sequence
Hypervariable Region 1	15971	Forward	5′-TTAACTCCACCATTAGCACC-3′
Hypervariable Region 1	16451	Reverse	5′-GCGAGGAGAGTAGCACTCTTG-3′
Hypervariable Region 2	15	Forward	5′-CACCCTATTAACCACTCACG-3′
Hypervariable Region 2	484	Reverse	5′-TGAGATTAGTAGTATGGGAG-3′

EXO-SAP

The EXO-SAP solution mainly consists of Exonuclease I and Shrimp Alkaline Phosphatase, which are hydrolytic enzymes. The Exonuclease I will degrade the remaining single-stranded primers and any accidental single-stranded DNA that were produced by the PCR. From the PCR mixture, there will be residual dNTPs that will interfere with the sequencing reaction, and the Shrimp Alkaline Phosphatase will hydrolyze those dNTPs. Therefore, after the PCR reaction is finished, the EXO-SAP solution is added to the PCR products (Appendix I). We have used the MultiGene Thermal Cycler to incubate the EXO-SAP tubes for 15 minutes at 37°C for treatment and another 15 minutes at 80°C for inactivation.

Appendix I

EXO-SAP Protocol

1. Wear the Post-PCR laboratory coat.
2. Wear a mask and double gloves.
3. Disinfect the inner part of the Compact Vertical Laminar Flow Bench(Telstar Mini-V/PCR) with 70% Isopropyl Alcohol wipes.
4. Disinfect the pipettes, the PCR racks, the outside of the tips boxes and the marker pen with 70% Isopropyl Alcohol wipes.
5. Remove the double gloves, and wear new double gloves. Then, wash the double gloved hands with 95% Ethanol solution.
6. Use the marker pen to label the EXO-SAP tube (on the top and the side of the tube) as HV1 with the sample code number. Take another EXO-SAP tube and label it as HV2 with the same sample code number. So, each sample must have two EXO-SAP tubes, one for HV1 and the other one for HV2.
7. Repeat step 6 for the next sample.
8. Dispense 2 µL of the EXO-SAP solution into each EXO-SAP tube.
9. Take 5 µL from each PCR reaction, and put them into their corresponding EXO-SAP tubes [according to their sample code numbers and Hypervariable regions].
10. Put the EXO-SAP tubes in the MultiGene Thermal Cycler. Run the "EXO-SAP" protocol, after typing the total volume = 7 µL. The "EXO-SAP" process will take 30 minutes.
11. After the "EXO-SAP" process is finished, place EXO-SAP tubes in the refrigerator at 4°C until the next step.

Gel Electrophoresis

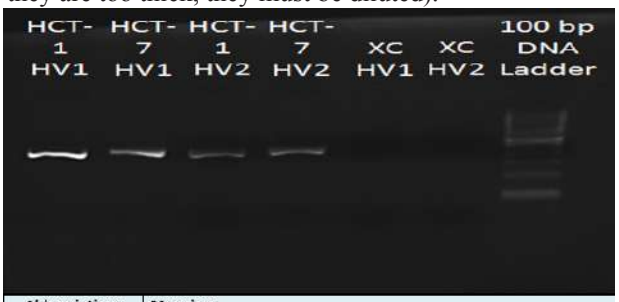
We have performed gel electrophoresis for the PCR products of Hypervariable Region 1 and Hypervariable Region 2 by using 3% Agarose gel, which is recommended for small PCR products of less than 150 nucleotides (Appendix J). The purpose of performing gel electrophoresis is to decide whether we should dilute the EXO-SAP tubes or not, based on the thickness of each band; if the bands are too thick, they must be diluted. This decision will tell us the exact amount that we should take from each EXO-SAP tube, in order to do the sequencing reaction, as explained in Figure 4-10.

Appendix J

Gel Electrophoresis Protocol

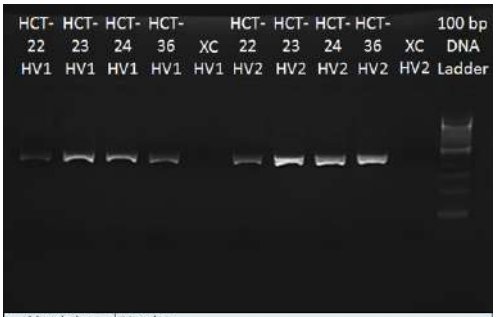
1. Wear the Post-PCR laboratory coat.
2. Wear a mask and double gloves.
3. Disinfect the working area, the pipettes and the outside of the tips boxes with 70% Isopropyl Alcohol wipes.
4. Place the pre-prepared 3% Agarose gel (3 g of Agarose powder + 100 mL of the 1X TAE (Tris-acetate-EDTA) buffer + 1 µL of the 10 mg/mL Ethidium Bromide (EtBr)) inside the electrophoresis chamber (in the center where the wells are near to the negative side and far away from the positive side of the electrophoresis).
5. Pour the right amount of 1X TAE buffer in the electrophoresis chamber (the right amount means that the gel is submerged under about 2 mm of buffer).
6. Add the desired number of tiny separated drops of the 10 mg/mL Ethidium Bromide on the Parafilm sheet (each drop of the 10 mg/mL Ethidium Bromide is for one PCR product).

7. For each PCR product ((HV1) and (HV2) PCR tubes), pipette 6 µL of the PCR product and dispense it into any drop of the 10 mg/mL Ethidium Bromide. Then, re-take it and mix it few times immediately. After that immediately load it into the well in the gel.
8. After loading all the wells, close the electrophoresis chamber with its lid.
9. Run the gel at 140 volts for 45 minutes.
10. After the running is finished, take out the gel and place it inside the Gene Flash Syngene Bio Imaging, which works with the Ultraviolet (UV) light.
11. After the photograph is displayed on the screen, decide the amount that we should take from each PCR product (which is present in the EXO-SAP tube) for the Sequencing step. The decision must consider the thickness of the bands (because if they are too thick, they must be diluted).



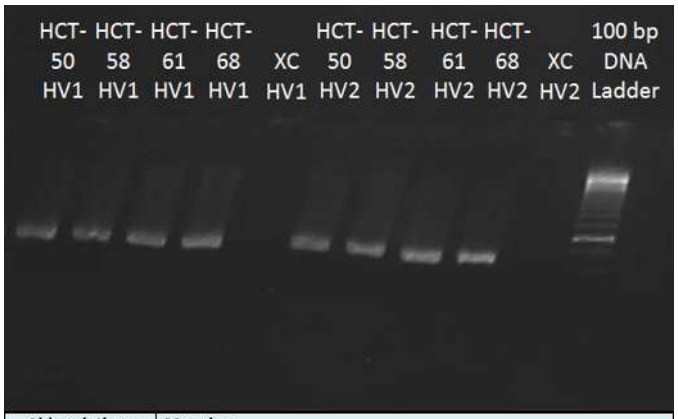
Abbreviations	Meanings
XC HV1	Extraction Negative Control for Hypervariable Region 1
XC HV2	Extraction Negative Control for Hypervariable Region 2
HV1	Hypervariable Region 1
HV2	Hypervariable Region 2
Sample Code No.	**The amount that should be moved to the Sequencing reaction**
HCT-1 HV1	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-7 HV1	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-1 HV2	Take directly 0.5 µL from the EXO-SAP tube.
HCT-7 HV2	Take directly 0.5 µL from the EXO-SAP tube.

Figure 4: Gel electrophoresis photograph for HCT-1 HV1, HCT-1 HV2, HCT-7 HV1 and HCT-7 HV2



Abbreviations	Meanings
XC HV1	Extraction Negative Control for Hypervariable Region 1
XC HV2	Extraction Negative Control for Hypervariable Region 2
HV1	Hypervariable Region 1
HV2	Hypervariable Region 2
Sample Code No.	**The amount that should be moved to the Sequencing reaction**
HCT-22 HV1	Take directly 0.5 µL from the EXO-SAP tube.
HCT-23 HV1	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-24 HV1	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-36 HV1	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-22 HV2	Take directly 0.5 µL from the EXO-SAP tube.
HCT-23 HV2	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-24 HV2	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-36 HV2	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.

Figure 5: Gel electrophoresis photograph for HCT-22 HV1, HCT-22 HV2, HCT-23 HV1, HCT-23 HV2, HCT-24 HV1, HCT-24 HV2, HCT-36 HV1 and HCT-36 HV2



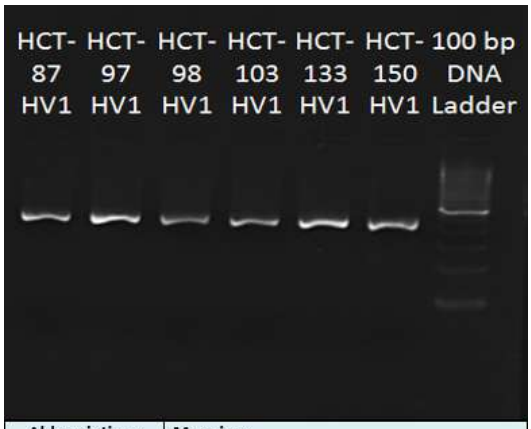
Abbreviations	Meanings
XC HV1	Extraction Negative Control for Hypervariable Region 1
XC HV2	Extraction Negative Control for Hypervariable Region 2
HV1	Hypervariable Region 1
HV2	Hypervariable Region 2
Sample Code No.	**The amount that should be moved to the Sequencing reaction**
HCT-50 HV1	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-58 HV1	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-61 HV1	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-68 HV1	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-50 HV2	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-58 HV2	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-61 HV2	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-68 HV2	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.

Figure 6: Gel electrophoresis photograph for HCT-50 HV1, HCT-50 HV2, HCT-58 HV1, HCT-58 HV2, HCT-61 HV1, HCT-61 HV2, HCT-68 HV1 and HCT-68 HV2

Abbreviations	Meanings
XC HV1	Extraction Negative Control for Hypervariable Region 1
XC HV2	Extraction Negative Control for Hypervariable Region 2
HV1	Hypervariable Region 1
HV2	Hypervariable Region 2
Sample Code No.	**The amount that should be moved to the Sequencing reaction**
HCT-69 HV1	Take directly 0.5 µL from the EXO-SAP tube.
HCT-70 HV1	Take directly 0.5 µL from the EXO-SAP tube.
HCT-71 HV1	Take directly 0.5 µL from the EXO-SAP tube.
HCT-72 HV1	Take directly 0.5 µL from the EXO-SAP tube.
HCT-74 HV2	Take directly 0.5 µL from the EXO-SAP tube.
HCT-69 HV2	Take directly 0.5 µL from the EXO-SAP tube.
HCT-70 HV2	Take directly 0.5 µL from the EXO-SAP tube.
HCT-71 HV2	Take directly 0.5 µL from the EXO-SAP tube.
HCT-72 HV2	Take directly 0.5 µL from the EXO-SAP tube.
HCT-74 HV2	Take directly 0.5 µL from the EXO-SAP tube.

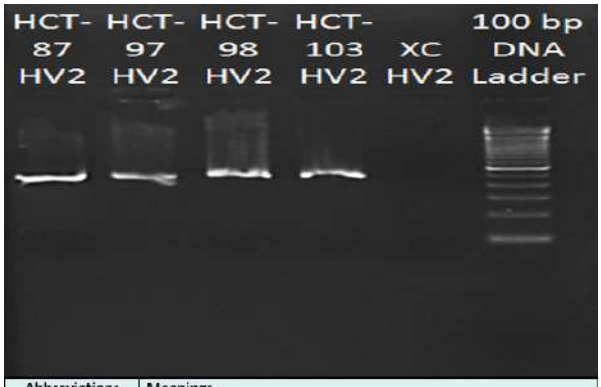
Figure 7: Gel electrophoresis photograph for HCT-69 HV1, HCT-69

HV2, HCT-70 HV1, HCT-70 HV2, HCT-71 HV1, HCT-71 HV2, HCT-72 HV1, HCT-72 HV2, HCT-74 HV1 and HCT-74 HV2



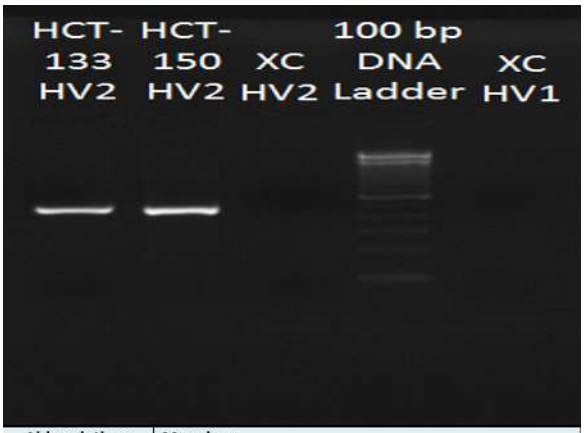
Abbreviations	Meanings
HV1	Hypervariable Region 1
Sample Code No.	**The amount that should be moved to the Sequencing reaction**
HCT-87 HV1	Take directly 0.5 µL from the EXO-SAP tube.
HCT-97 HV1	Take directly 0.5 µL from the EXO-SAP tube.
HCT-98 HV1	Take directly 0.5 µL from the EXO-SAP tube.
HCT-103 HV1	Take directly 0.5 µL from the EXO-SAP tube.
HCT-133 HV1	Take directly 0.5 µL from the EXO-SAP tube.
HCT-150 HV1	Take directly 0.5 µL from the EXO-SAP tube.

Figure 8: Gel electrophoresis photograph for HCT-87 HV1, HCT-97 HV1, HCT-98 HV1, HCT-103 HV1, HCT-133 HV1 and HCT-150 HV1



Abbreviations	Meanings
XC HV2	Extraction Negative Control for Hypervariable Region 2
HV2	Hypervariable Region 2
Sample Code No.	**The amount that should be moved to the Sequencing reaction**
HCT-87 HV2	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-97 HV2	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-98 HV2	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.
HCT-103 HV2	The EXO-SAP tube should be diluted 1:2 with Nuclease-Free water. Then, take 0.5 µL from the diluted EXO-SAP tube.

Figure 9: Gel electrophoresis photograph for HCT-87 HV2, HCT-97 HV2, HCT-98 HV2 and HCT-103 HV2



Abbreviations	Meanings
XC HV2	Extraction Negative Control for Hypervariable Region 2
HV2	Hypervariable Region 2
Sample Code No.	**The amount that should be moved to the Sequencing reaction**
HCT-133 HV2	Take directly 0.5 µL from the EXO-SAP tube.
HCT-150 HV2	Take directly 0.5 µL from the EXO-SAP tube.

Figure 10: Gel electrophoresis photograph for HCT-87 HV2, HCT-97 HV2, HCT-98 HV2 and HCT-103 HV2

Sequencing

The sequencing reaction is done by the Sanger method for DNA sequencing by using Big Dye X Terminator Cycle Sequencing kit from the Applied Biosystems® (Appendix K). The sequencing reaction differs from the PCR reaction in one thing that is the addition of the chain terminators, which are the dideoxyribonucleotide triphosphates (ddNTPs). Those are capable of generating fragments of single nucleotide resolution, as described in Figure 11.

Appendix K

Sequencing Protocol

1. Wear the Pre-PCR laboratory coat.
2. Wear a mask and double gloves.
3. Disinfect the inner part of the PCR WorkStation with 70% Isopropyl Alcohol wipes.
4. Disinfect the pipettes, the PCR racks, the outside of the tips boxes and the marker pen with 70% Isopropyl Alcohol wipes.
5. Remove the double gloves, and wear new double gloves. Then, wash the double gloved hands with 95% Ethanol solution.
6. Use the marker pen to label the PCR tube (on the top and the side of the tube) as HV1 F (Forward) with the sample code number. Take a second PCR tube and label it as HV1 R (Reverse) with the same sample code number. Take a third PCR tube and label it as HV2 R (Forward) with the same sample code number. Take a fourth PCR tube and label it as HV2 R (Reverse) with the same sample code number. So, each sample must have four PCR tubes, two for HV1 (Forward and Reverse) and the other two for HV2 (Forward and Reverse).
7. Repeat step 6 for the next sample.
8. Dispense 5.25 µL of Nuclease-Free Water, 2 µL of RRM (Ready Reaction Mix), 1 µL of Sequencing buffer and 1.25 µL of Forward Primer [HV1] into each (HV1 F) PCR tube.
9. Dispense 5.25 µL of Nuclease-Free Water, 2 µL of RRM (Ready Reaction Mix), 1 µL of Sequencing buffer and 1.25 µL of Reverse Primer [HV1] into each (HV1 R) PCR tube.
10. Dispense 5.25 µL of Nuclease-Free Water, 2 µL of RRM (Ready Reaction Mix), 1 µL of Sequencing buffer and 1.25 µL of

Forward Primer [HV2] into each (HV2 F) PCR tube.

11. Dispense 5.25 µL of Nuclease-Free Water, 2 µL of RRM (Ready Reaction Mix), 1 µL of Sequencing buffer and 1.25 µL of Reverse Primer [HV2] into each (HV2 R) PCR tube.
12. Remove the Pre-PCR laboratory coat (to enter the Post-PCR section) and wear the Post-PCR laboratory coat for the Post-PCR section.
13. Wear a mask and double gloves.
14. Disinfect the inner part of the Compact Vertical Laminar Flow Bench(Telstar Mini-V/PCR) with 70% Isopropyl Alcohol wipes.
15. Disinfect the pipettes, the PCR racks, the outside of the tips boxes and the marker pen with 70% Isopropyl Alcohol wipes.
16. Remove the double gloves, and wear new double gloves. Then, wash the double gloved hands with 95% Ethanol solution.
17. Take 0.5 µL from each EXO-SAP tube (from the original or the diluted EXO-SAP tube according to the gel electrophoresis), and put them into their corresponding PCR tubes [according to their sample code numbers and Hypervariable regions].
18. Centrifuge the (HV1 F), (HV1 R), (HV2 F) and (HV2 R) PCR tubes for 1 minute at 14.1 rcf [Relative Centrifugal Force].
19. Put the (HV1 F), (HV1 R), (HV2 F) and (HV2 R) PCR tubes in the MultiGene Thermal Cycler. Run the "Cycle Sequencing" protocol, after typing the total volume = 10 µL. The "Cycle Sequencing" process will take 2 hours and 37 minutes.
20. After the "Cycle Sequencing" process is finished, place the (HV1 F), (HV1 R), (HV2 F) and (HV2 R) PCR tubes in the refrigerator at 4°C until the next step.

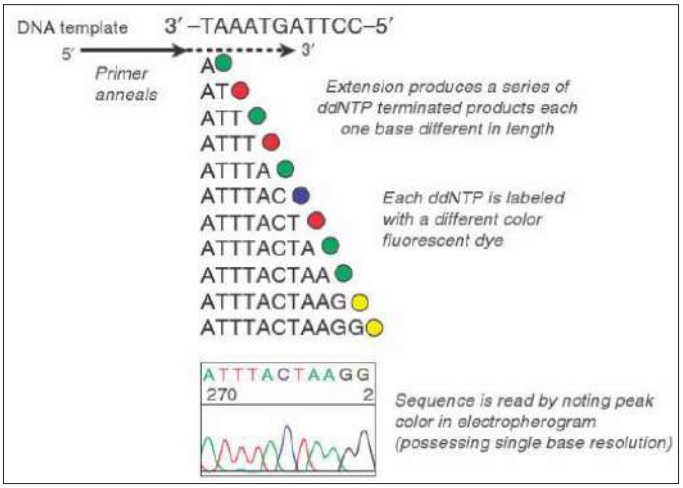


Figure 11: The Sanger sequencing process. With one primer, the sequencing reaction splits into separate reactions by the ddNTPs that are labeled with four different colored fluorescent dyes [5]

Clean-Up

After the sequencing reaction is finished, a Clean-up step should be encountered before loading the samples into the ABI PRISM®310 Genetic Analyzer capillary autosequencer. The Clean-up step is done by using the Big Dye X Terminator Purification kit from the Applied Biosystems® (Appendix L).This kit contains two solutions, which are X Terminator and SAM solutions that are capable of isolating the free salts, dNTPs and unincorporated dye terminators at the bottom of a microcentrifuge tube, so by this technique, it will prevent them from being co-injected with the dye-labeled extension fragments from the sequencing reaction. The ABI PRISM®310 Genetic Analyzer capillary autosequencer is a single capillary electrophoresis, the negatively charged DNA will be moved through the capillary that is filled with a viscous liquid gel (POP-7) by an electrokinetic injection to the positive electrode. According to the size of the fragments, a laser beam will be excited on the smallest fragment first and will be detected by the fluorescent detector, and the largest fragment will be last one to be detected, Figure 12 shows the inner parts of the ABI PRISM®310 Genetic Analyzer capillary autosequencer.

Appendix L

Clean-up Protocol

1. Wear the Post-PCR laboratory coat.
2. Wear a mask and double gloves.
3. Disinfect the inner part of the Compact Vertical Laminar Flow Bench (Telstar Mini-V/PCR) with 70% Isopropyl Alcohol wipes.
4. Disinfect the pipettes, the PCR racks, the outside of the tips boxes and the marker pen with 70% Isopropyl Alcohol wipes.
5. Remove the double gloves, and wear new double gloves. Then, wash the double gloved hands with 95% Ethanol solution.
6. Use the marker pen to label the microcentrifuge tube (on the top only) as HV1 F (Forward) with the sample code number. Take a second microcentrifuge tube and label it as HV1 R (Reverse) with the same sample code number. Take a third microcentrifuge tube and label it as HV2 R (Forward) with the same sample code number. Take a fourth microcentrifuge tube and label it as HV2 R (Reverse) with the same sample code number. So, each sample must have four microcentrifuge tubes, two for HV1 (Forward and Reverse) and the other two for HV2 (Forward and Reverse).
7. Repeat step 6 for the next sample.
8. Dispense 10 µL of Big Dye X Terminator solution and 45 µL of SAM solution into each labeled microcentrifuge tube.
9. Take 10 µL from each sequenced DNA, and put them into their corresponding labeled microcentrifuge tubes [according to their sample code numbers, Hypervariable regions and the type of the primer].
10. Mix the labeled microcentrifuge tubes by using the ThermoMixer at room temperature for 35 minutes at 1400 rpm [Revolutions Per Minute].
11. At the meantime, use the marker pen to label the Genetic Analyzer tubes as the labeled microcentrifuge tubes (each Genetic Analyzer tube represents one labeled microcentrifuge tube).
12. After the ThermoMixer is finished, centrifuge the labeled microcentrifuge tubes for 2 minutes at 14.1 rcf [Relative Centrifugal Force].
13. After the centrifugation is finished, take 20 µL of the supernatant from each labeled microcentrifuge tube, and put them into their corresponding labeled Genetic Analyzer tubes [according to their sample code numbers, Hypervariable regions and the type of the primer].
14. Secure the Genetic Analyzer tubes with Genetic Analyzer septa.
15. Place the secured labeled Genetic Analyzer tubes in the ABI PRISM®310 Genetic Analyzer capillary autosequencer. Each Genetic Analyzer tube will take 1 hour to get processed. Use the ABI PRISM® SeqScape® Software Version 2.6 to create a "Mutation Report", which will contain the profile for all the specimens.

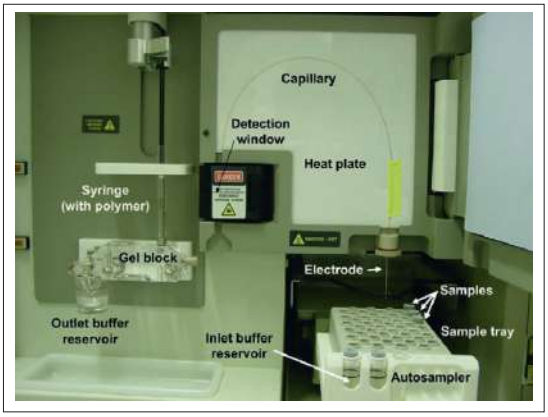


Figure 12: Photograph of the inner parts of the ABI PRISM®310 Genetic Analyzer capillary autosequencer with a single capillary, for a single sequencing reaction, the electrophoresis separation takes 1 hour to be processed [3]

Results

We have used the ABI PRISM® SeqScape® Software Version 2.6 to create a "Mutation Report", which will contain the mitochondrial DNA profile for all the 21 specimens (Appendix M). We have created a list of the polymorphisms that are needed to be BioEdited due to the presence of insertions (Appendix N).

Appendix M

Mutation Report

SeqScape 2.6.0 Mutation Report

Generated at: 30 Apr 2015 at 12:35:23 GST

Summary

Active Layer	Layer 1	Project	HCT
Project Creation Date	07 Apr 2015 at 09:38:04 GST	Project Modification Date	23 Apr 2015 at 14:17:39 GST
Project Template (PT)	MITO	PT Creation Date	25 Nov 2013 at 13:30:03 GST
PT Modification Date	23 Apr 2015 at 14:17:39 GST	Reference Date Group (RDG)	MITO
RDG Creation Date	25 Nov 2013 at 13:19:58 GST	RGD Modification Date	23 Apr 2015 at 14:17:39 GST
Display Settings Date	MITO	DS Creation Date	25 Nov 2013 at 13:24:56 GST
DS Modification Date	23 Apr 2015 at 14:17:39 GST	Analysis Defaults (AD)	MITO
AD Creation Date	25 Nov 2013 at 13:28:48 GST	AD Modification Date	23 Apr 2015 at 14:17:39 GST

Specimens in Report
103HV1, 103HV2, 133HV1, 133HV2, 150HV1, 150HV2, 1HV1, 1HV2, 22HV1, 22HV2, 23HV1, 23HV2, 24HV1, 24HV2, 36HV1, 36HV2, 50HV1, 50HV2, 58HV1, 58HV2, 61HV1, 61HV2, 68HV1, 68HV2, 69HV1, 69HV2, 70HV1, 70HV2, 71HV1, 71HV2, 72HV1, 72HV2, 74HV1, 74HV2, 7HV1, 7HV2, 86HV1, 86HV2, 97hv1, 97hv2, 98HV1, 98HV2

Mutations

Specimen	Base Change	ROI	Position	Length	Type	QV	Known	Effect	Aa Change
103HV1	16093t>C	r CRS	16093	1	Sub	36	no	missense	F5365L
103HV1	16192c>T	r CRS	16192	1	Sub	48	no	missense	P5398S
103HV1	16201c>T	r CRS	16201	1	Sub	41	no	missense	Q5401*
103HV1	16223c>T	r CRS	16223	1	Sub	46	no	missense	P5408L
103HV2	73a>G	r CRS	73	1	Sub	47	no	missense	M25V
103HV2	189a>G	r CRS	189	1	Sub	46	no	silent	-
103HV2	195t>C	r CRS	195	1	Sub	44	no	silent	-

103HV2	204t>C	r CRS	204	1	Sub	46	no	silent	-
103HV2	207g>A	r CRS	207	1	Sub	45	no	silent	-
103HV2	210a>G	r CRS	210	1	Sub	46	no	silent	-
103HV2	263a>G	r CRS	263	1	Sub	46	no	missense	H88R
103HV2	315-316insC	r CRS	315	1	Ins	48	no	frameshift insertion	-
133HV1	16223c>T	r CRS	16223	1	Sub	48	no	missense	P5408L
133HV1	16240a>G	r CRS	16240	1	Sub	48	no	missense	N5414D
133HV2	146t>C	r CRS	146	1	Sub	48	no	missense	L49P
133HV2	235a>G	r CRS	235	1	Sub	37	no	missense	I79V
133HV2	263a>G	r CRS	263	1	Sub	41	no	missense	H88R
133HV2	315-316insC	r CRS	315	1	Ins	47	no	frameshift insertion	-
150HV1	16126t>C	r CRS	16126	1	Sub	45	no	missense	Y5376H
150HV1	16185c>T	r CRS	16185	1	Sub	47	no	silent	-
150HV1	16355c>T	r CRS	16355	1	Sub	37	no	missense	P5452L
150HV1	16362t>C	r CRS	16362	1	Sub	42	no	silent	-
150HV2	58t>C	r CRS	58	1	Sub	20	no	missense	F20L
150HV2	64c>T	r CRS	64	1	Sub	44	no	silent	-
150HV2	146t>C	r CRS	146	1	Sub	45	no	missense	L49P
150HV2	263a>G	r CRS	263	1	Sub	35	no	missense	H88R
150HV2	310t>C	r CRS	310	1	Sub	37	no	missense	
150HV2	310-311insTC	r CRS	310	2	Ins	34 (avg)	no	frameshift insertion	-
1HV1	15983t>A	r CRS	15983	1	Sub	12	no	missense	-
1HV1	16223c>T	r CRS	16223	1	Sub	47	no	missense	P5405L
1HV2	235a>G	r CRS	235	1	Sub	42	no	missense	I79V
1HV2	263a>G	r CRS	263	1	Sub	41	no	missense	H88R
1HV2	315-316insC	r CRS	315	1	Ins	45	no	frameshift insertion	-
22HV1	16069c>T	r CRS	16069	1	Sub	50	no	missense	H5357Y
22HV1	16093t>C	r CRS	16093	1	Sub	38	no	missense	F5365L
22HV1	16126t>C	r CRS	16126	1	Sub	44	no	missense	Y5376H
22HV1	16145g>A	r CRS	16145	1	Sub	47	no	silent	-
22HV1	16222c>T	r CRS	16222	1	Sub	47	no	missense	P5408S
22HV1	16261c>T	r CRS	16261	1	Sub	44	no	missense	P5421S
22HV1	16300a>G	r CRS	16300	1	Sub	43	no	missense	T5434A
22HV2	73a>G	r CRS	73	1	Sub	36	no	missense	M25V
22HV2	183a>G	r CRS	183	1	Sub	48	no	silent	-
22HV2	263a>G	r CRS	263	1	Sub	44	no	missense	H88R
22HV2	295c>T	r CRS	295	1	Sub	45	no	missense	P99S
22HV2	315-316insC	r CRS	315	1	Ins	48	no	frameshift insertion	-
22HV2	462c>T	r CRS	462	1	Sub	40	no	missense	S154L
23HV1	16093t>C	r CRS	16093	1	Sub	36	no	missense	F5365L
23HV1	16148c>T	r CRS	16148	1	Sub	47	no	missense	P5383L
23HV1	16172t>C	r CRS	16172	1	Sub	42	no	missense	I5391T
23HV1	16187c>T	r CRS	16187	1	Sub	47	no	missense	P5396L

23HV1	16188c>G	r CRS	16188	1	Sub	47	no	missense	P5396L
23HV1	16189t>C	r CRS	16189	1	Sub	45	no	missense	S5397P
23HV1	16223c>T	r CRS	16223	1	Sub	48	no	missense	P5408L
23HV1	16230a>G	r CRS	16230	1	Sub	45	no	silent	-
23HV1	16311t>C	r CRS	16311	1	Sub	42	no	silent	-
23HV1	16320c>T	r CRS	16320	1	Sub	43	no	silent	-
23HV2	64c>T	r CRS	64	1	Sub	28	no	silent	-
23HV2	93a>G	r CRS	93	1	Sub	48	no	silent	-
23HV2	152t>C	r CRS	152	1	Sub	47	no	missense	L51P
23HV2	189a>G	r CRS	189	1	Sub	38	no	silent	-
23HV2	204t>C	r CRS	204	1	Sub	44	no	silent	-
23HV2	207g>A	r CRS	207	1	Sub	40	no	silent	-
23HV2	236t>C	r CRS	236	1	Sub	46	no	missense	I79T
23HV2	247g>A	r CRS	247	1	Sub	48	no	missense	E83K
23HV2	263a>G	r CRS	263	1	Sub	44	no	missense	H88R
23HV2	315-316insC	r CRS	315	1	Ins	47	no	frameshift insertion	-
24HV1	16051a>G	r CRS	16051	1	Sub	38	no	missense	T5351A
24HV1	16093t>C	r CRS	16093	1	Sub	38	no	missense	F5365L
24HV1	16209t>C	r CRS	16209	1	Sub	48	no	silent	-
24HV1	16239c>T	r CRS	16239	1	Sub	47	no	silent	-
24HV1	16305a>G	r CRS	16305	1	Sub	47	no	silent	-
24HV1	16352t>C	r CRS	16352	1	Sub	43	no	missense	I5451T
24HV1	16353c>T	r CRS	16353	1	Sub	46	no	missense	I5451T
24HV2	26c>A	r CRS	26	1	Sub	13	no	missense	T9N
24HV2	73t>C	r CRS	73	1	Sub	39	no	missense	M25V
24HV2	146t>C	r CRS	146	1	Sub	49	no	missense	L49P
24HV2	152t>C	r CRS	152	1	Sub	45	no	missense	L51P
24HV2	234a>G	r CRS	234	1	Sub	45	no	missense	I78M
24HV2	263a>G	r CRS	263	1	Sub	46	no	missense	H88R
24HV2	315-316insC	r CRS	315	1	Ins	47	no	frameshift insertion	-
36HV1	16219a>G	r CRS	16219	1	Sub	47	no	missense	N5407D
36HV1	16261c>T	r CRS	16261	1	Sub	44	no	missense	P5421S
36HV2	146t>C	r CRS	146	1	Sub	46	no	missense	L49P
36HV2	263a>G	r CRS	263	1	Sub	43	no	missense	H88R
36HV2	310t>C	r CRS	310	1	Sub	39	no	missense	-
36HV2	310-311insTC	r CRS	310	2	Ins	42 (avg)	no	frameshift insertion	-
50HV1	16051a>G	r CRS	16051	1	Sub	36	no	missense	T5351A
50HV1	16278c>T	r CRS	16278	1	Sub	38	no	silent	-
50HV2	30t>A	r CRS	30	1	Sub	7	no	missense	-
50HV2	143g>A	r CRS	143	1	Sub	40	no	missense	C48Y
50HV2	263a>G	r CRS	263	1	Sub	44	no	missense	H88R
50HV2	309c>T	r CRS	309	1	Sub	47	no	silent	-
50HV2	310t>C	r CRS	310	1	Sub	43	no	missense	-
50HV2	310-311insTC	r CRS	310	2	Ins	47 (avg)	no	frameshift insertion	-

58HV1	16278c>T	r CRS	16278	1	Sub	39	no	silent	-
58HV2	73a>G	r CRS	73	1	Sub	36	no	missense	M25V
58HV2	263a>G	r CRS	263	1	Sub	40	no	missense	H88R
58HV2	310t>C	r CRS	310	1	Sub	43	no	missense	-
58HV2	310-311insTC	r CRS	310	2	Ins	43 (avg)	no	frameshift insertion	-
61HV1	16069c>T	r CRS	16069	1	Sub	44	no	missense	H535Y
61HV1	16145g>A	r CRS	16145	1	Sub	44	no	silent	-
61HV1	16261c>T	r CRS	16261	1	Sub	43	no	missense	P5421S
61HV2	60delt	r CRS	60	1	Del	14 (avg)	no	frameshift deletion	-
61HV2	73a>G	r CRS	73	1	Sub	34	no	missense	M25C
61HV2	151c>T	r CRS	151	1	Sub	45	no	missense	L51H
61HV2	152t>C	r CRS	152	1	Sub	47	no	missense	L51H
61HV2	263a>G	r CRS	263	1	Sub	47	no	missense	H88A
61HV2	295c>T	r CRS	295	1	Sub	45	no	missense	P99H
61HV2	310t>C	r CRS	310	1	Sub	44	no	missense	-
61HV2	310-311insTC	r CRS	310	2	Ins	45 (avg)	no	frameshift insertion	-
61HV2	462c>T	r CRS	462	1	Sub	42	no	missense	S154L
68HV1	16213g>a	r CRS	16213	1	Sub	45	no	missense	A5405T
68HV1	16224t>C	r CRS	16224	1	Sub	48	no	silent	-
68HV1	16301c>T	r CRS	16301	1	Sub	44	no	missense	T5434I
68HV1	16311t>C	r CRS	16311	1	Sub	41	no	silent	-
68HV2	73a>G	r CRS	73	1	Sub	30	no	missense	M25V
68HV2	146t>C	r CRS	146	1	Sub	45	no	missense	L49P
68HV2	152t>C	r CRS	152	1	Sub	43	no	missense	L51P
68HV2	263a>G	r CRS	263	1	Sub	33	no	missense	H88R
68HV2	310t>C	r CRS	310	1	Sub	33	no	missense	-
68HV2	310-311insTC	r CRS	310	2	Ins	36 (avg)	no	frameshift insertion	-
69HV1	16069c>T	r CRS	16069	1	Sub	41	no	missense	H5357Y
69HV1	16093t>C	r CRS	16093	1	Sub	41	no	missense	F5365L
69HV1	16126t>C	r CRS	16126	1	Sub	44	no	missense	Y5376H
69HV1	16145g>A	r CRS	16145	1	Sub	45	no	silent	-
69HV1	16222c>T	r CRS	16222	1	Sub	47	no	missense	P5408S
69HV1	16261c>T	r CRS	16261	1	Sub	45	no	missense	P5421S
69HV1	16300a>G	r CRS	16300	1	Sub	43	no	missense	T5434A
69HV2	73a.G	r CRS	73	1	Sub	43	no	missense	M25V
69HV2	263a>G	r CRS	263	1	Sub	45	no	missense	H88R
69HV2	295c>T	r CRS	295	1	Sub	44	no	missense	P99S
69HV2	315-316insC	r CRS	315	1	Ins	48	no	frameshift insertion	-
69HV2	462c>T	r CRS	462	1	Sub	50	no	missense	S154L
70HV1	16126t>C	r CRS	16126	1	Sub	38	no	missense	Y5376H
70HV1	16163a>G	r CRS	16163	1	Sub	18	no	missense	K5388R
70HV1	16186c>T	r CRS	16186	1	Sub	40	no	missense	P5396S
70HV1	16189t>C	r CRS	16189	1	Sub	39	no	missense	S5397P

70HV1	[16193-16193delC]+[=]	r CRS	16193	1	HIM	15	no	heterozygous deletion	-
70HV1	16263t>c	r CRS	16263	1	Sub	6	no	silent	-
70HV1	16263-16264insT	r CRS	16263	1	Ins	36	no	frameshift insertion	-
70HV1	16294c>T	r CRS	16294	1	Sub	48	no	missense	P5432I
70HV2	73a>G	r CRS	73	1	Sub	34	no	missense	M25V
70HV2	263a>G	r CRS	263	1	Sub	44	no	missense	H88R
70HV2	310t>C	r CRS	310	1	Sub	45	no	missense	-
70HV2	310-311insTC	r CRS	310	2	Ins	48 (avg)	no	frameshift insertion	-
71HV1	16260c>T	r CRS	16260	1	Sub	47	no	silent	-
71HV1	16399a>G	r CRS	16399	1	Sub	34	no	missense	T5467A
71HV2	152t>C	r CRS	152	1	Sub	41	no	missense	L51P
71HV2	263a>G	r CRS	263	1	Sub	36	no	missense	H88R
71HV2	310t>C	r CRS	310	1	Sub	38	no	missense	S104P
71HV2	312-313insTCCC	r CRS	312	4	Ins	33 (avg)	no	frameshift insertion	-
71HV2	438-439insC	r CRS	438	1	Sub	22	no	frameshift insertion	-
72HV1	16366c>T	r CRS	16366	1	Sub	45	no	missense	H5456Y
72HV2	55t>C	r CRS	55	1	Sub	24	no	missense	Y19L
72HV2	56a>T	r CRS	56	1	Sub	44	no	missense	Y19L
72HV2	57t>C	r CRS	57	1	Sub	29	no	missense	Y19L
72HV2	263a>G	r CRS	263	1	Sub	35	no	missense	H88R
72HV2	310t>C	r CRS	310	1	Sub	43	no	missense	S104P
72HV2	310-311insCTC	r CRS	310	3	Ins	38 (avg)	no	frameshift insertion	-
74HV1	16126t>C	r CRS	16126	1	Sub	47	no	missense	Y5376H
74HV1	16234c>T	r CRS	16234	1	Sub	44	no	missense	H5412Y
74HV1	16355c>T	r CRS	16355	1	Sub	43	no	missense	P5452L
74HV1	16362t>C	r CRS	16362	1	Sub	44	no	silent	-
74HV2	146t>C	r CRS	146	1	Sub	46	no	missense	L49P
74HV2	263a>G	r CRS	263	1	Sub	42	no	missense	H88R
74HV2	309-310insCT	r CRS	309	2	Ins	36 (avg)	no	frameshift insertion	-
74HV2	310deit	r CRS	310	1	Del	36 (avg)	no	frameshift deletion	-
74HV2	310-311insC	r CRS	310	1	Ins	43	no	frameshift insertion	-
7HV1	15956t>G	r CRS	15956	1	Sub	26	no	missense	I5319K
7HV1	15957c>A	r CRS	15957	1	Sub	34	no	missense	I5319K
7HV1	15959g>C	r CRS	15959	1	Sub	34	no	missense	R5320E
7HV1	15963a>G	r CRS	15963	1	Sub	30	no	missense	E5321Q
7HV1	15966a>T	r CRS	15966	1	Sub	17	no	missense	K5322R
7HV1	15967g>A	r CRS	15967	1	Sub	16	no	missense	V5323I
7HV1	15968t>G	r CRS	15968	1	Sub	15	no	missense	V5323I
7HV1	15969c>T	r CRS	15969	1	Sub	19	no	missense	V5323I
7HV1	15972t>A	r CRS	15972	1	Sub	8	no	missense	F5324V

7HV1	15981-15982insC	r CRS	15981	1	Ins	19	no	frameshift insertion	-
7HV1	16051a>G	r CRS	16051	1	Sub	27	no	missense	T5351A
7HV1	16278c>T	r CRS	16278	1	Sub	36	no	silent	-
7HV2	73a>G	r CRS	73	1	Sub	42	no	missense	M25V
7HV2	263a>G	r CRS	263	1	Sub	44	no	missense	H88R
7HV2	310t>C	r CRS	310	1	Sub	38	no	missense	-
7HV2	310-311insTC	r CRS	310	2	Ins	40 (avg)	no	frameshift insertion	-
86HV1	16188c>T	r CRS	16188	1	Sub	45	no	silent	-
86HV1	16223c>T	r CRS	16223	1	Sub	45	no	missense	P5408L
86HV1	16231t>C	r CRS	16231	1	Sub	47	no	missense	S5411P
86HV1	16362t>C	r CRS	16362	1	Sub	43	no	silent	-
86HV1	16390g>A	r CRS	16390	1	Sub	50	no	missense	G5464S
86HV2	73a>G	r CRS	73	1	Sub	42	no	missense	M25V
86HV2	146t>C	r CRS	146	1	Sub	49	no	missense	L49P
86HV2	263a>G	r CRS	263	1	Sub	46	no	missense	H88R
86HV2	315-316insC	r CRS	315	1	Ins	47	no	frameshift insertion	-
86HV2	461c>T	r CRS	461	1	Sub	34	no	missense	S154L
97hv1	46051a>G	r CRS	16051	1	Sub	31	no	missense	T5351A
97hv1	16278c>T	r CRS	16278	1	Sub	44	no	silent	-
97hv2	73a>G	r CRS	73	1	Sub	32	no	missense	M25V
97hv2	200a>G	r CRS	200	1	Sub	44	no	silent	-
97hv2	263a>G	r CRS	263	1	Sub	42	no	missense	H88R
97hv2	309-310insCT	r CRS	309	2	Ins	44 (avg)	no	frameshift insertion	-
97hv2	310delt	r CRS	310	1	Del	44 (avg)	no	frameshift deletion	-
97hv2	310-311insC	r CRS	310	1	Ins	44	no	frameshift insertion	-
98HV1	16051a>G	r CRS	16051	1	Sub	39	no	missense	T5351A
98HV1	16145g>A	r CRS	16145	1	Sub	46	no	silent	-
98HV1	16206a>C	r CRS	16206	1	Sub	47	no	silent	-
98HV1	16319g>A	r CRS	16319	1	Sub	45	no	missense	S5440N
98HV2	73a>G	r CRS	73	1	Sub	35	no	missense	M25V
98HV2	146t>C	r CRS	146	1	Sub	48	no	missense	L49P
98HV2	150c>T	r CRS	150	1	Sub	48	no	silent	-
98HV2	152t>C	r CRS	152	1	Sub	45	no	missense	L51P
98HV2	194c>T	r CRS	194	1	Sub	45	no	missense	T65I
98HV2	195t>C	r CRS	195	1	Sub	45	no	missense	T65I
98HV2	263a>G	r CRS	263	1	Sub	40	no	missense	H88R
98HV2	315-316insC	r CRS	315	1	Ins	46	no	frameshift insertion	-

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Appendix N

BioEdit List

No.	Specimen	Base Change	Position	Length	Effect	BioEdit
1	103HV2	315-316insC	315	1	frameshift insertion	315.1C
2	133HV2	315-316insC	315	1	frameshift insertion	315.1C
3	133HV2	310-311insTC	310	2	frameshift insertion	310.1T-310.2C
4	1HV2	315-316insC	315	1	frameshift insertion	315.1C
5	22HV2	315-316insC	315	1	frameshift insertion	315.1C
6	23HV2	315-316insC	315	1	frameshift insertion	315.1C
7	24HV2	315-316insC	315	1	frameshift insertion	315.1C
8	36HV2	310-311insTC	310	2	frameshift insertion	310.1T-310.2C
9	50HV2	310-311insTC	310	2	frameshift insertion	310.1T-310.2C
10	58HV2	310-311insTC	310	2	frameshift insertion	310.1T-310.2C
11	61HV2	60delt	60	1	frameshift deletion	60DEL
12	61HV2	310-311insTC	310	2	frameshift insertion	310.1T-310.2C
13	68HV2	310-311insTC	310	2	frameshift insertion	310.1T-310.2C
14	69HV2	315-316insC	315	1	frameshift insertion	315.1C
15	70HV1	[16193- 16193delC]+[=]	16193	1	heterozygous deletion	16193DEL
16	70HV1	16263-16264insT	16263	1	frameshift insertion	16263.1T
17	70HV2	310-311insTC	310	2	frameshift insertion	310.1T-310.2C
18	71HV2	312-313 insTCCC	312	4	frameshift insertion	312.1T-312.2C- 312.3C-312.4C
19	71HV2	438-439insC	438	1	frameshift insertion	438.1C
20	72HV2	310-311insCTC	310	3	in-frame insertion	310.1C-310.2T-310.3C
21	74HV2	309-310insCT	309	2	frameshift insertion	309.1C-310.2T
22	74HV2	310delt	310	1	frameshift deletion	310DEL
23	74HV2	310-311insC	310	1	frameshift insertion	310.1C
24	7HV1	15981-15982insC	15981	1	frameshift insertion	15981.1C
25	7HV2	310-311insTC	310	2	frameshift insertion	310.1T-310.2C
26	86HV2	315-316insC	315	1	frameshift insertion	315.1C
27	97HV2	309-310insCT	309	2	frameshift insertion	309.1C-309.2T
28	97HV2	310delt	310	1	frameshift deletion	315DEL
29	97HV2	310-311insC	310	1	frameshift insertion	310.1C
30	98HV2	315-316insC	315	1	frameshift insertion	315.1C

Discussion

Two regions only from the whole mitochondrial DNA were selected to do our study on, which are Hypervariable Region 1 that is 481 base pair long and Hypervariable Region 2 that is 469 base pair long. Our study was on 21 unrelated UAE female students that were 20 year old to 25 year old, and some of them live in Sharjah, Ajman, Umm Al Quwain, Dubai and Ras Al Khaimah. Those 21 students were originally Emiratis, which means that their maternal grandmothers were also Emiratis. We have created mitochondrial DNA profiles for the 21 students, as shown in Table 3 and Table 4, we called them as haplotype data. A total of 229 polymorphisms were observed carefully from those haplotype data. Among the 229 polymorphisms, 93 polymorphisms were in Hypervariable Region 1 and 136 polymorphisms were in Hypervariable Region 2, as illustrated in Figure 13. Therefore, polymorphisms are more likely to occur more in Hypervariable Region 2 than Hypervariable Region 1. Out of the 229 polymorphisms, we have identified 106 different polymorphisms. Then, from the 106 different polymorphisms, we classified them into unique and common polymorphisms, as explained in Figure 14. In Figure 15, there is a list of the 34 common polymorphisms with their frequencies to each of the 21 students. The term "Common Polymorphism" means that if there is a polymorphism that 2 individuals or more share it, we call it as a common polymorphism, which is not necessary all the 21 students should have it. Interestingly, there is a polymorphism at position 263 from Adenine to Guanine that all the 21 students have it, as described in Figure 15. Moreover, there are unique polymorphisms that represent each individual of the 21 students, which are listed in Table 5. However, out of the 21 students, two of them don't have any unique polymorphism, and they are HCT-58 and HCT-69.

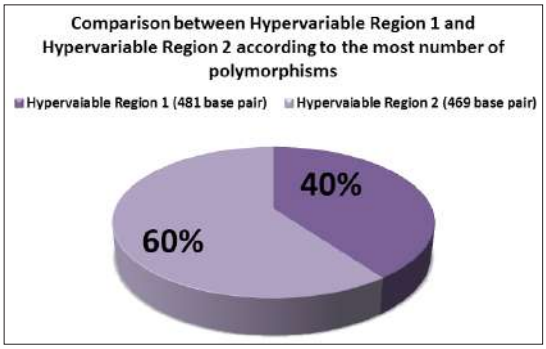

Figure 13: Comparison between Hypervariable Region 1 and Hypervariable Region 2 according to the most number of polymorphisms. 60% of Hypervariable Region 2 were polymorphic, while 40% of Hypervariable Region 1 were polymorphic

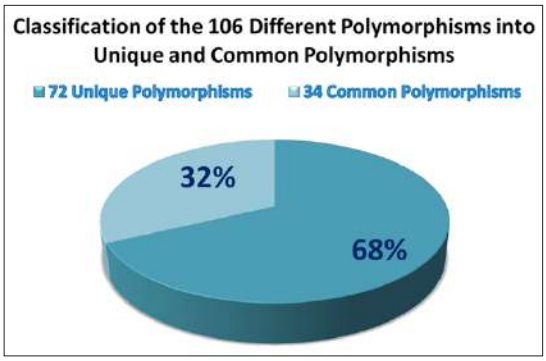

Figure 14: The classification of the 106 different polymorphisms into unique and commonpolymorphisms. Unique polymorphisms are unique representatives for each family name, whereas, common polymorphisms are the ones that are found in each family name

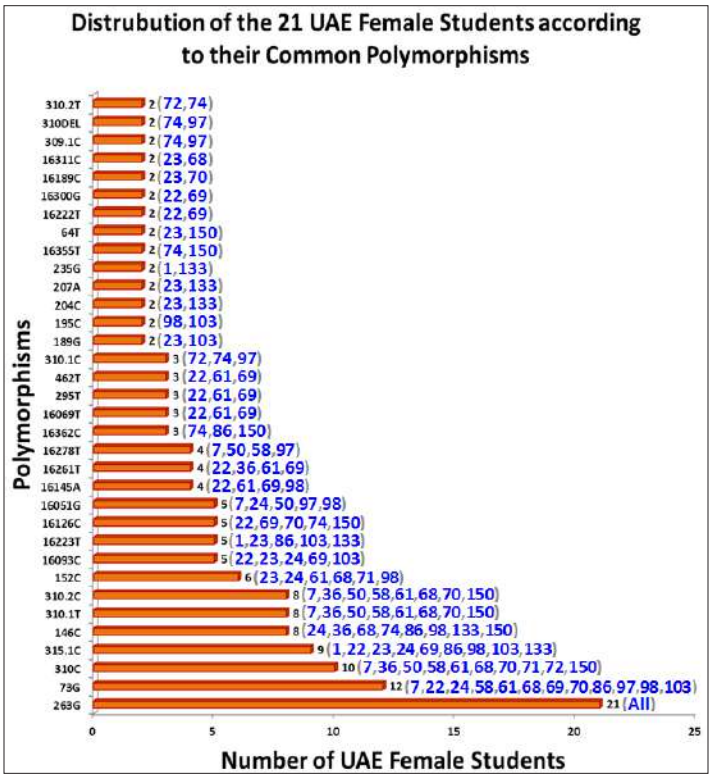

Figure 15: The distribution of the 21 UAE female students according to their common polymorphisms. Between the brackets are the last numbers of the code number thatrepresents each student. For example, (72,74) means that HCT-72 and HCT-74

Table 3: The Haplotype Data of HCT-1, HCT-7, HCT-22, HCT-23, HCT-24, HCT-36, HCT-50, HCT-58, HCT-61, HCT-68 and HCT-69

Code No.	Family Name*	Residence	Region of Mitochondrial Genome**	Haplotype Data***
HCT-1	AlKaabi	Umm Al Quwain	Hypervariable region 1	15983A–16223T
			Hypervariable region 2	235G–263G–315.1C
HCT-7	AlKetbi	Sharjah	Hypervariable region 1	15956G–15957A–15959C–15963G–15966T–15967A–15968G–15969T–15972A–15981.1C–16051G–16278T
			Hypervariable region 2	73G–263G–310C–310.1T–310.2C
HCT-22	AlOwais	Sharjah	Hypervariable region 1	16069T–16093C–16126C–16145A–16222T–16261T–16300G
			Hypervariable region 2	73G–183G–263G–295T–315.1C–462T
HCT-23	AlShamsi	Umm Al Quwain	Hypervariable region 1	16093C–16148T–16172C–16187T–16188G–16189C–16223T–16230G–16311C–16320T
			Hypervariable region 2	64T–93G–152C–189G–204C–207A–236C–247A–263G–315.1C
HCT-24	AlKetbi	Sharjah	Hypervariable region 1	16051G–16093C–16209C–16239T–16305G–16352C–16353T
			Hypervariable region 2	26A–73G–146C–152C–234G–263G–315.1C
HCT-36	AlMejlad	Umm Al Quwain	Hypervariable region 1	16219G–16261T
			Hypervariable region 2	146C–263G–310C–310.1T–310.2C
HCT-50	AlShamsi	Ajman	Hypervariable region 1	16051G–16278T
			Hypervariable region 2	30A–143A–263G–309T–310C–310.1T–310.2C

HCT-58	AlKhasoni	Sharjah	Hypervariable region 1	16278T
			Hypervariable region 2	73G–263G–310C–310.1T–310.2C
HCT-61	AlSuwaidi	Ajman	Hypervariable region 1	16069T–16145A–16261T
			Hypervariable region 2	60Deletion–73G–151T–152C–263G–295T–310C–310.1T–310.2C–462T
HCT-68	AlDafri	Sharjah	Hypervariable region 1	16213A–16224C–16301T–16311C
			Hypervariable region 2	73G–146C–152C–263G–310C–310.1T–310.2C
HCT-69	AlSuwaidi	Sharjah	Hypervariable region 1	16069T–16093C–16126C–16145A–16222T–16261T–16300G
			Hypervariable region 2	73G–263G–295T–315.1C–462T

*The family name of the sample's maternal grandmother.

**The Mitochondrial Genome has many regions, and in our case, we are targeting only two regions (Hypervariable region 1 and Hypervariable region 2).

***A profile that contains a set of polymorphisms, which tends to be inherited from a single parent (In case of mtDNA, it is a maternal inheritance).

Table 4: The Haplotype Data of HCT-70, HCT-71, HCT-72, HCT-74, HCT-86, HCT-97, HCT-98, HCT-103, HCT-133 and HCT-15

Code No.	Family Name*	Residence	Region of Mitochondrial Genome**	Haplotype Data***
HCT-70	AlFalasi	Ajman	Hypervariable region 1	16126C–16163G–16186T–16189C–16193Deletion–16263C–16263.1T–16294T
			Hypervariable region 2	73G–263G–310C–310.1T–310.2C
HCT-71	AlTamimi	Ras Al Khaimah	Hypervariable region 1	16260T–16399G
			Hypervariable region 2	152C–263G–310C–312.1T–312.2C–312.3C–312.4C–438.1C
HCT-72	AlKaabi	Sharjah	Hypervariable region 1	16366T
			Hypervariable region 2	55C–56T–57C–263G–310C–310.1C–310.2T–310.3C
HCT-74	AlKaabi	Ajman	Hypervariable region 1	16126C–16234T–16355T–16362C
			Hypervariable region 2	146C–263G–309.1C–310.2T–310Deletion–310.1C
HCT-86	AlSuwaidi	Dubai	Hypervariable region 1	16188T–16223T–16231C–16362C–16390A
			Hypervariable region 2	73G–146C–263G–315.1C–461T
HCT-97	AlAleeli	Umm Al Quwain	Hypervariable region 1	16051G–16278T
			Hypervariable region 2	73G–200G–263G–309.1C–309.2T–310Deletion–310.1C
HCT-98	AlSuwaidi	Ajman	Hypervariable region 1	16051G–16145A–16206C–16319A
			Hypervariable region 2	73G–146C–150T–152C–194T–195C–263G–315.1C
HCT-103	AlShamsi	Sharjah	Hypervariable region 1	16093C–16192T–16201T–16223T
			Hypervariable region 2	73G–189G–195C–204C–207A–210G–263G–315.1C
HCT-133	AlSuwaidi	Umm Al Quwain	Hypervariable region 1	16223T–16240G
			Hypervariable region 2	146C–235G–263G–315.1C
HCT-150	AlAleeli	Ajman	Hypervariable region 1	16126C–16185T–16355T–16362C
			Hypervariable region 2	58C–64T–146C–263G–310C–310.1T–310.2C

*The family name of the sample's maternal grandmother.

**The Mitochondrial Genome has many regions, and in our case, we are targeting only two regions (Hypervariable region 1 and Hypervariable region 2).

***A profile that contains a set of polymorphisms, which tends to be inherited from a single parent (In case of mtDNA, it is a maternal inheritance).

Table 5: Unique Polymorphisms that represent each individual

Code No.	Unique Polymorphisms
HCT-1	15983A
HCT-7	15956G-15957A-15959C-15963G-15966T-15967A-15968G-15969T-15972A-15981.1C
HCT-22	183G
HCT-23	16148T-16172C-16187T-16188G-16230G-16320T-93G-236C-247A
HCT-24	16209C-16239T-16305G-16352C-16353T-26A-234G
HCT-36	16219G
HCT-50	30A-143A-309T
HCT-58	None*
HCT-61	151T-60Deletion
HCT-68	16213A-16224C-16301T
HCT-69	None**
HCT-70	16163G-16186T-16263C-16294T-16263.1T-16193Deletion
HCT-71	16260T-16399G-312.1T-312.2C-312.3C-312.4C-438.1C
HCT-72	16366T-55C-56T-57C-310.3C
HCT-74	16234T
HCT-86	16188T-16231C-16390A-461T
HCT-97	200G-309.2T
HCT-98	16206C-16319A-150T-194T
HCT-103	16192T-16201T-210G
HCT-133	16240G
HCT-150	16185T-58C

*Because all HCT-58's polymorphisms were found in HCT-7's common polymorphisms, and with other families, but with different proportions.

** Because all HCT-69's polymorphisms were found in HCT-22's common polymorphisms, and with other families, but with different proportions.

Is it possible that HCT-58 and HCT-69 are from the "Established Group" that the other 19 students came from them, and even if they were from the "Established Group", why they still preserve their mitochondrial DNA and why they did not establish any unique polymorphism?

Butler declares that because of the high rate of mutations in the mitochondrial DNA, comparing individuals of the same maternal line could be so difficult depends on if those individuals were distant maternal relatives or close maternal relatives [5]. Butler adds, mitochondrial DNA tests were performed between Tsarina Alexandra and Prince Philip, who is the third generation of Tsarina Alexandra, and between Tsar Nicholas II and Xenia Cheremeteff-Sfiri, who is the fourth generation of Tsar Nicholas II, and the tests revealed that there is a 100% match between Tsarina Alexandra and Prince Philip, and 99% match between Tsar Nicholas II and Xenia Cheremeteff-Sfiri [5]. This means that comparison of mitochondrial DNA that is more than three generations old is more likely to get at least one mutation in the current generation. Therefore, if we did a vertical study on HCT-58 and HCT-69 with their older generations, we will find out that HCT-58 and HCT-69 have unique polymorphisms compared to their older generations.

"Skipping Generations" is a term that I have invented to solve the mystery of having two individuals with no unique polymorphisms compared to the other 19 students. For instance, the grandmother represents the 1st generation, the mother represents the 2nd generation and the granddaughter represents the 3rd generation, in normal circumstances, the aunt is close to the mother's generation in terms of age, but sometimes that doesn't happen. For example, instead, the aunt is actually close to the granddaughter's generation in terms of age, this phenomenon is called as Skipping Generations, as explained in Figure 16. In that case, whoever relates to the aunt will have less unique polymorphisms than those who relate to the granddaughter. Therefore, HCT-58 and HCT-69 are effects of Skipping Generations phenomenon. Our research reveals that there is a commonality between the 21 unrelated UAE female students.

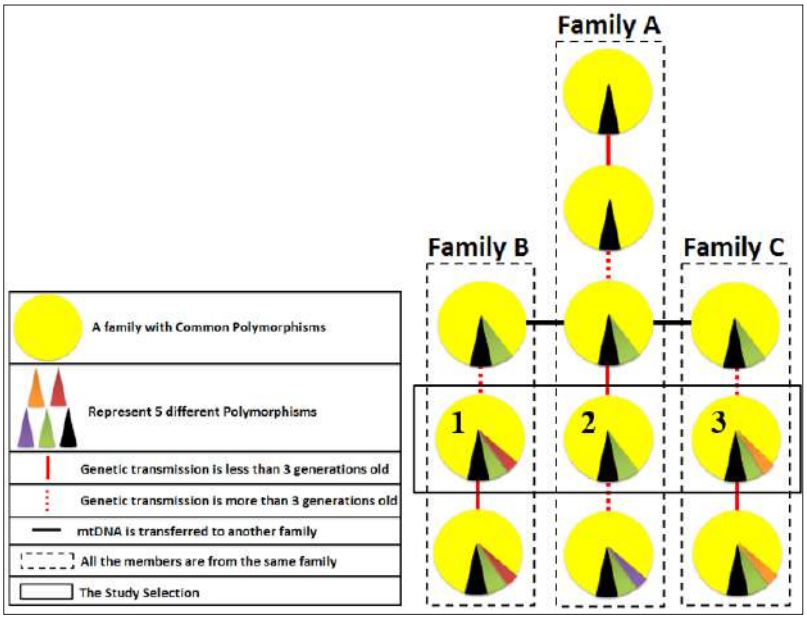

Figure 16: Skipping Generations. The study selection illustrates three different individuals that represent three different families, but all of them are close to each other in terms of age. Number 1 and Number 3 have their own unique polymorphisms, except for Number 2 who doesn't have any unique polymorphism compared to Number 1 and Number 3. Because Number 1 and Number 3 are from the same generation, while Number 2 actually relates to an older generation

Conclusion

It's impossible for anyone to preserve their mtDNA from their great ancestors till now. The discovery of the remains of the Romanov family back in 1991 concluded that the comparison of mtDNA that is more than three generations old is more likely to get at least one mutation in the current generation. Therefore, if a vertical study is done on those two individuals with their older generations, definitely they will have unique polymorphisms compared to their older generations. Those two individuals are the effect of "Skipping Generations" phenomenon, the term that I have invented to solve the mystery of having two individuals with no unique polymorphisms.

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