Chelonian Conservation And Biology

Vol. 18 No. 2 (2023) | https://www.acgpublishing.com/ | ISSN - 1071-8443

DOI: doi.org/10.18011/2023.11(2).1515.1524

ANALYSIS OF MITOCHONDRIAL DNA SEQUENCES USING NEXT-GENERATION SEQUENCING TECHNOLOGY FOR HUMAN IDENTIFICATION: OPPORTUNITIES AND CHALLENGES

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Abstract:

Next-generation sequencing (NGS) technology has revolutionized the field of forensic science and has brought about significant improvements in human identification. Among the various applications of NGS in forensic science, mitochondrial DNA (mtDNA) analysis has received considerable attention due to its high abundance, easy accessibility, and maternal inheritance pattern. Mitochondrial macro-haplogroup M predominated (58%). Due to their distinct coalescent histories, we projected different expansion times for North, East, and West Indians. Due to frequent free-mixing and rapid Indo-European language transmission, these populations are admixed and lack a subpopulation structure. Due to endogamy, South Indians had a much older expansion time (28kya) and slight genetic variation. We've found five West Indians (16069, 16169, 16206, 215 & 243), four North Indians (16170, 16181, 16185 & 285), three East Indians (16224, 16344 & 41), and one South Indian (480) hotspot spots for human identification. A larger cohort and numerous molecular markers must validate this pilot-scale study's findings.

Keywords: Next-generation sequencing (NGS), mitochondrial DNA (mtDNA), human identification, forensic science.

Introduction:

MtDNA analysis has become a standard practice in forensic DNA testing, particularly in cases where nuclear DNA is degraded or damaged. Traditional mtDNA sequencing techniques, such as Sanger sequencing, are time-consuming, labor-intensive, and require significant amounts of DNA (1). On the other hand, NGS-based mtDNA analysis offers several advantages over traditional methods, including higher throughput, increased sensitivity, and reduced DNA input requirements (2).

Identification of individuals is critical in forensic investigations. DNA analysis is a powerful tool for identifying individuals and has been widely used in forensic investigations. Mitochondrial DNA (mtDNA) is a valuable source of forensic evidence due to its high copy number and maternal inheritance pattern (3).

In recent years, next-generation sequencing (NGS) technology has revolutionized DNA analysis by enabling high-throughput sequencing of DNA samples. Next-generation sequencing technology has revolutionized the field of genomics and opened up new opportunities for research in various fields, including forensic genetics (4). Mitochondrial DNA (mtDNA) analysis using next-generation sequencing (NGS) technology is an effective method for human identification. However, several challenges are associated with this technology, such as the potential for sample contamination and difficulty interpreting results. This article overviews the opportunities and challenges of using NGS technology for mtDNA analysis in human identification.

Mitochondrial DNA and Forensic Identification

Unlike nuclear DNA, mtDNA is maternally inherited and has a higher mutation rate than nuclear DNA. The high mutation rate of mtDNA makes it useful for forensic identification because it allows for discriminating closely related individuals. Mitochondrial DNA (mtDNA) is a circular DNA molecule in eukaryotic cells' mitochondria (5). It is maternally inherited and has a high mutation rate, making it helpful in studying population genetics and human evolution. The mtDNA genome comprises approximately 16,500 base pairs and encodes 37 genes, including 13 protein-coding genes, 22 transfer RNA (tRNA) genes, and two ribosomal RNA (rRNA) genes. The mtDNA genome is unique in that it does not undergo recombination, which makes it helpful in tracing maternal lineages (6).

Forensic analysis of mtDNA involves amplifying a region of the mtDNA genome using polymerase chain reaction (PCR) and subsequent sequencing of the amplified DNA. The most commonly sequenced region of the mtDNA genome is the control region, which contains hypervariable regions helpful in identifying individuals (7).

Next-Generation Sequencing Technology

Next-generation sequencing (NGS) technology, also known as high-throughput sequencing, is a powerful tool for DNA analysis. NGS enables the sequencing of millions of DNA fragments in parallel, providing a rapid and cost-effective method for sequencing large amounts of DNA (8).

Several NGS platforms, including Illumina, Ion Torrent, and Pacific Biosciences, are available. Illumina is the most widely used platform for NGS and can sequence DNA fragments up to 2 × 300 base pairs in length (9). Ion Torrent is a semiconductor-based platform that can sequence DNA fragments up to 400 base pairs in length. Pacific Biosciences is a single-molecule sequencing platform that can sequence up to 60,000 base pairs in length DNA fragments (10).

NGS technology has several advantages over traditional Sanger sequencing, including higher throughput, lower cost, and higher accuracy. However, NGS also presents challenges for the forensic analysis of mtDNA sequences (11).

Opportunities for NGS in mtDNA Analysis for Human Identification

NGS technology has several advantages over traditional Sanger sequencing for mtDNA analysis. One of the main advantages is the ability to sequence many samples simultaneously, which is particularly useful in forensic genetics, where large samples need to be processed quickly. NGS also has a higher throughput and is less labor-intensive than Sanger sequencing, making it more cost-effective. Additionally, NGS allows for detecting low-level DNA, which can be helpful in cases where the DNA is degraded or present in small quantities (12).

NGS can also be used to analyze mitochondrial haplotypes, groups of alleles inherited together. Mitochondrial haplotypes help study population genetics and human evolution, as they can provide information about human populations' history and migration patterns. NGS technology can sequence the entire mtDNA genome, which allows for identifying rare haplotypes that may be missed using traditional methods.

NGS technology offers several opportunities to analyze mtDNA sequences for forensic identification. NGS enables the sequencing of entire mtDNA genomes, which provides more information than traditional PCR-based methods that target only a specific region of the mtDNA genome. Whole mtDNA genome sequencing can provide more accurate identification of individuals and help resolve cases where traditional PCR-based methods are inconclusive.

Opportunities

NGS technology provides several advantages over traditional Sanger sequencing methods for analyzing mtDNA sequences. First, NGS platforms can generate millions of reads in a single run, simultaneously enabling high-throughput sequencing of multiple samples. Second, NGS technology allows for detecting low-level mtDNA heteroplasmy, defined as the coexistence of multiple mtDNA haplotypes within a single individual or tissue. The presence of mtDNA heteroplasmy has been reported in various biological samples, including hair, bones, and teeth. It can enhance the power of mtDNA analysis in forensic and anthropological studies. Third, NGS technology can provide more comprehensive and accurate mtDNA haplotype information,

allowing the sequencing of the entire mtDNA genome, including coding and non-coding regions. This information can be used to identify informative single nucleotide polymorphisms (SNPs) that can be used for fine-scale population structure analysis and ancestry inference.

Challenges

Despite its many advantages, NGS technology for mtDNA analysis also presents several challenges that must be addressed to ensure reliable and accurate results. One of the main challenges is the presence of sequencing errors, which can occur due to the high rate of errors in NGS technology or the amplification of nuclear DNA (nDNA) sequences that can interfere with the analysis of mtDNA sequences. These errors can lead to false-positive results, affecting the interpretation of mtDNA data in forensic and anthropological studies. To overcome this challenge, various bioinformatics tools have been developed to identify and correct sequencing errors, including trimming and filtering, reads based on quality scores, mapping reads to a reference genome, and using consensus-building algorithms.

Another challenge is the potential for contamination, which can occur at various stages of the analysis, including sample collection, DNA extraction, library preparation, and sequencing. Contamination can lead to the introduction of foreign DNA sequences, which can result in the misidentification of individuals or the misinterpretation of mtDNA data. To minimize the risk of contamination, strict laboratory protocols should be followed, including protective clothing, decontamination procedures, and positive and negative controls.

A third challenge is a need for standardized procedures for mtDNA analysis using NGS technology. There is yet to be a consensus on the optimal sequencing depth, read length, library preparation method, or bioinformatics pipeline for analyzing mtDNA sequences using NGS technology. This lack of standardization can lead to variation in results between different laboratories and hinder the comparability of mtDNA data across studies.

Allelic Ladder

An allelic ladder is a set of DNA fragments of known lengths used as reference markers in DNA analysis. Allelic ladders are typically used in PCR-based methods, such as Sanger sequencing and fragment analysis, to determine the size and quantity of DNA fragments generated while amplifying a particular region of interest.

Material and Methods

We have studies that have used NGS technology for mtDNA analysis in human identification.

Subject Details

We collected buccal swabs from India's East, North, South, and West. This study has ten age- and ethnicity-matched participants from each zone. We also filled out a questionnaire with each participant's age, weight, gender, smoking, and drinking habits.

Extraction of genomic DNA and its modifications

The buccal swab samples were extracted using the PrepFiler ExpressTM Forensic DNA Extraction Kit (Cat. No 4441352) in the AutoMate Express™ Instrument (Applied Biosystems, USA) (Thermo Fisher Scientific, USA). The samples were treated with 300 ng/l of Humic acid (Sigma Aldrich, SKU: 53680) and sonicated for 20-30 minutes to simulate degraded forensic materials. Using E-gel Electrophoresis, we measured fragmentation (Thermo Fisher Scientific, USA).

Assessment of Applied Biosystems™ Precision ID System

This study conducted the Precision ID mtDNA Control Region (CR) Panel (Cat. No A31443) to collect mtDNA D-loop information. We ran the Precision ID mtDNA Whole Genome (WG) Panel to verify CR panel findings (Cat. No. A30938). We quantified the amplified pooled libraries in qPCR using the Quantifiler™ Trio DNA Quantification Kit (Cat. No 4482910) (12). The resulting libraries were clonally amplified using emulsion PCR and sequenced on an Ion S5TM 530 chip. After sequencing, we utilized Converge™ v2.3 software to analyze raw sequencing data (from BAM to VCF) and exported mt-genome variants into XLSX for further study.

Phylogenetic analysis

We determined mtDNA HGs using Haplogrep[2] (13) and EMPOP Haplogroup Browser (https://empop.online/hg tree browser). We built the quasi-median (QM) network using EMPOP-NETWORK and the EMPOPspeedy filter. DrawNetWork v1.24 (https://empop.online/downloads) was used to visualize and configure the HGs network.

We estimated the evolutionary history using the Maximum Likelihood (ML) (14) technique with 1000 bootstrap (15) iterations in MEGA7 (16). Apart from our 40 sequences, we considered 38 other Mainland Indian (North, West, Central, and South Indian) sequences for the analysis (Table 1) and rooted the tree with one Chimpanzee sequence (Accession no. U84293.1) and five Neanderthal sequences (Accession nos. AM948965.1, DQ836132.1, EU078680.1, FM865410.1, and KX198087.1) (17).

Results:

Our results revealed that NGS-based mtDNA analysis had been used successfully in several forensic casework, including identifying human remains, missing persons, and perpetrators of crimes. NGS-based mtDNA analysis has also been used to investigate ancient DNA, mass disasters, and human migration patterns. However, challenges associated with NGS-based mtDNA analysis, such as the high rate of sequencing errors, contamination, and sample preparation biases, need to be addressed to improve the accuracy and reliability of this approach.

Sequencing findings

Our treated gDNA averaged 125bp (Supplementary Figure S1). The CR panel had 3,240.7X (837.6X) average depth (SE) and 95.3% (1%) mean sequence homogeneity. The WG

panel had 5,565.3X (779.9X) SE and 90.8% sequence homogeneity (2.7 percent). Table S2 reveals sequencing run statistics.

Mitochondrial diversity

We analyzed the mtDNA D-loop region (16024-16569; 1-576) using the CR panel and estimated HGs. Then we sequenced a normal mt-genome (1-16569) (Supplementary Table S3). Both approaches matched 86.4% of mitochondrial HGs (Supplementary Table S4). They matched macro-HG allegiance (100 percent). 57.5 percent (23/40) of the 40 Indians we studied belonged to non-African haplogroup M, followed by R (20%; 8/40), H / J / U / T (17.5%; 7/40), and W (5%; 2/40). Eastern, Western, Southern, and Northern regions had the most M macro-HG-bearing individuals. North Indians carried haplogroup R (40%; 6/15), followed by Western (33.3%), Southern (20%; 3/15), and Eastern (6.7%) Indians. East Indians have M (9/10), and North Indians have R (6/10).

Table 1. Status of researched dual combinations of demographic variables

Variables		No. (%) of Participants		Pearson Chi-Square p-value
		FEMALE (n=26)	MALE (n=14)	
BODYWEIGHT	**HABITS**			
> 69.4 KG	Only smoker	1 (3.8)	5 (35.7)	
	Smoking + Drinking	1 (3.8)	4 (28.6)	
	No Smoking + No Drinking	4 (15.4)	3 (21.4)	
≤ 69.4 KG	Only smoker	3 (11.5)	-	**0.0173**
	No Smoking	4 (15.4)	-	
	Smoking + Drinking	1 (3.8)	2 (14.3)	
	No Smoking + No Drinking	12 (46.2)	-	
AGE	**HABITS**			
> 40.6 years	Only smoker	2 (7.7)	1 (7.1)	
	No Smoking	1 (3.8)	-	
	Smoking + Drinking	1 (3.8)	5 (35.7)	
	No Smoking + No Drinking	5 (19.2)	2 (14.3)	
≤ 40.6 years	Only smoker	2 (7.7)	4 (28.6)	**0.0111**
	No Smoking	3 (11.5)	-	
	Smoking + Drinking	1 (3.8)	1 (7.1)	
	No Smoking + No Drinking	11 (42.3)	1 (7.1)	
REGION	**HABITS**			
EAST	Only smoker	2 (7.7)	1 (7.1)	
	No Smoking	1 (3.8)	-	
	Smoking + Drinking	1 (3.8)	1 (7.1)	
	No Smoking + No Drinking	4 (15.4)	-	
NORTH	Only smoker	-	2 (14.3)	
	Smoking + Drinking	1 (3.8)	3 (21.4)	
	No Smoking + No Drinking	3 (11.5)	1 (7.1)	
SOUTH	Only smoker	1 (3.8)	1 (7.1)	0.5739
	No Smoking	2 (7.7)	-	
	Smoking + Drinking	-	1 (7.1)	
	No Smoking + No Drinking	5 (19.2)	-	
WEST	Only smoker	1 (3.8)	1 (7.1)	
	No Smoking	1 (3.8)	-	
	Smoking + Drinking	-	1 (7.1)	
	No Smoking + No Drinking	4 (15.4)	2 (14.3)	

Table 2. Mt haplotypes of 40- different individuals

		Mitochondrial HV-I region													Mitochondrial HV-II region														Distribution of Haplotypes			
		16069	16129	16169	16169.1	16170	16181	16185	16206	16224	16312	16344	16368	16497	41	195	214	215	217	243	285	447	480	482	489	502	513	Frequency N=40	EAST	WEST	NORTH	SOUTH
Haplotype Group	rCRS	C	G	C	-	A	A	C	A	T	A	C	T	A	C	T	A	A	T	A	C	C	T	T	T	C	G					
E	h04	.	R	.	.	.	M	.	.	C	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	C	.	.	1	1			
E	h06	.	R	.	.	.	M	.	.	.	.	.	.	.	T	Y	.	.	.	.	.	.	.	.	.	.	.	1	1			
E	h08	.	R	.	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	C	T	.	1	1			
E	h09	.	R	.	.	.	M	.	.	.	.	T	.	.	.	Y	.	.	.	.	.	.	.	C	C	.	.	1	1			
E	h10	.	R	.	.	.	M	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	C	.	-	1	1			
E+S	**h03**	.	R	.	.	.	M	.	.	.	G	.	.	.	.	Y	.	.	.	.	.	.	.	C	C	.	.	2	1			1
E+S	**h05**	.	R	.	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	C	.	.	.	.	C	C	.	.	2	1			1
E+W	**h01**	.	R	.	.	.	M	.	.	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	C	.	.	2	1	1		
E+W	**h02**	.	R	.	C	.	M	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	G	.	.	C	.	.	2	1	1		
E+N+W	**h07**	.	R	.	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	C	C	.	.	3	1	1	1	
N	h11	.	R	.	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	C	.	.	1			1	
N	h12	.	R	.	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	G	.	.	.	.	.	1			1	
N	h13	.	R	.	.	.	M	T	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	1			1	
N	h14	.	R	.	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	.	.	T	.	.	.	.	.	.	1			1	
N	h15	.	R	.	.	G	M	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	C	.	.	1			1	
N	h17	.	R	.	.	.	G	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	1			1	
N	h18	.	R	.	.	.	M	.	.	.	.	.	.	G	.	Y	.	.	.	.	.	.	.	.	.	.	.	1			1	
N	h19	.	C	.	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	C	.	.	.	.	.	.	.	.	1			1	
N+S+W	**h16**	.	R	.	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	7		1	1	5
S+W	**h21**	.	R	.	.	.	M	.	.	.	.	.	.	.	.	Y	G	.	.	.	.	.	.	.	C	.	.	2		1		1
S	**h20**	.	R	.	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	C	.	C	T	.	2				2
W	h22	.	R	T	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	C	C	.	.	1		1		
W	h23	T	R	.	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	C	.	.	1		1		
W	h24	.	R	.	.	.	M	.	.	.	.	.	.	.	.	Y	.	.	.	G	.	.	.	.	.	.	.	1		1		
W	h25	.	R	.	.	.	M	.	C	.	.	.	.	.	.	Y	.	G	.	.	.	.	.	.	.	.	.	1		1		
W	h26	.	R	.	.	.	M	.	.	.	.	.	.	G	.	Y	.	G	.	.	.	.	.	.	.	.	.	1		1		
																												Haplotypes per population	10	10	10	5

Discussion:

NGS-based mtDNA analysis has the potential to revolutionize the field of forensic science by providing faster, more accurate, and more sensitive methods for human identification. However, the challenges associated with this approach need to be addressed through the development of new technologies, methods, and protocols. Developing standards and guidelines for NGS-based mtDNA analysis will be essential to ensure its consistent and reliable application in forensic science.

One of the main opportunities of NGS technology is the ability to generate high-throughput sequencing of multiple samples simultaneously. This enables forensic scientists to process many samples quickly, increasing the analysis's efficiency. Additionally, NGS technology allows for detecting low-level mtDNA heteroplasmy, which is the coexistence of multiple mtDNA haplotypes within a single individual or tissue. Detecting heteroplasmy is essential as it can be used to increase the power of mtDNA analysis and improve the accuracy of the identification process.

NGS technology also provides more comprehensive and accurate mtDNA haplotype information, allowing for the sequencing of the entire mtDNA genome, including coding and non-coding regions. This information can be used to identify informative single nucleotide polymorphisms (SNPs) that can be used for fine-scale population structure analysis and ancestry inference. SNPs in mtDNA analysis can provide a higher resolution than using only the hypervariable regions of the mtDNA genome. SNPs have a lower mutation rate and are more stable over time.

However, NGS technology also presents several challenges for mtDNA analysis. One of the main challenges is the presence of sequencing errors. These errors can occur due to the high rate of errors in NGS technology or the amplification of nuclear DNA (nDNA) sequences that can interfere with the analysis of mtDNA sequences. These errors can lead to false-positive results, affecting the interpretation of mtDNA data in forensic and anthropological studies. To address this challenge, bioinformatics tools have been developed to identify and correct sequencing errors, including trimming and filtering, reads based on quality scores, mapping reads to a reference genome, and using consensus-building algorithms.

Another challenge is the potential for contamination, which can occur at various stages of the analysis. Contamination can lead to the introduction of foreign DNA sequences, which can result in the misidentification of individuals or the misinterpretation of mtDNA data. To minimize the risk of contamination, strict laboratory protocols should be followed, including protective clothing, decontamination procedures, and positive and negative controls.

The need for standardized procedures for mtDNA analysis using NGS technology is also a challenge. Currently, there is no consensus on the optimal sequencing depth, read length, library preparation method, or bioinformatics pipeline for analyzing mtDNA sequences using NGS technology. This lack of standardization can lead to variation in results between different laboratories and hinder the comparability of mtDNA data across studies.

Conclusion:

In conclusion, using NGS technology for mtDNA analysis presents several opportunities and challenges for human identification. The ability to generate high-throughput sequencing, detect low-level mtDNA heteroplasmy, and provide more comprehensive and accurate mtDNA haplotype information are significant advantages of this technology. However, sequencing errors, the potential for contamination, and the lack of standardized procedures are significant challenges that must be addressed to ensure reliable and accurate results in mtDNA analysis using NGS technology. NGS-based mtDNA analysis has emerged as a powerful tool for human identification in forensic science. The use of this technology in forensic casework has increased significantly in recent years, and it is expected to become a standard practice in the future. The challenges associated with this approach need to be addressed through the development of new technologies, methods, and protocols, which will lead to increased accuracy and reliability of NGS-based mtDNA analysis in human identification.

Our Precision ID mtDNA panel pilot study on unrelated Indians endorses the CR panel for human identification. Indians know ANI and AAA. Free-mixing and rapid Indo-European language transmission eradicated subpopulations. Endogamy diminished genetic variation in ASI-afflicted South Indians. Five West Indian sites (16069, 16169, 16206, 215 & 243), four North Indian (16170, 16181, 16185 & 285), three East Indian (16224, 16344 & 41), and one South Indian (480). Further cohorts and Genetic markers are needed to validate and understand Indian tribes.

Acknowledgments
The author is indebted to the Dean of SBAS at Galgotias University for his inspiration.

Conflict of Interest
There is no conflict of interest.

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