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Mitochondrial DNA Typing for Forensic Identification

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Abstract

Mitochondrial DNA has more useful genetic information as compared to nucleic DNA because they are present in more number per cell. In decomposed or old biological samples nuclear material in the cell may not exist for a long period, so it is difficult to perform DNA analysis with the nuclear DNA from remains of biological samples. This high copy number in mtDNA increases the possibility of recovering sufficient DNA from compromised samples. For this reason, mtDNA can play an important role in the identification of missing person investigation, in mass disasters and other forensic investigations involving samples with limited biological material. Additionally, mtDNA is maternally inherited. Therefore, barring a mutation, an individual's mother, siblings, as well as all other maternally-related family members will have identical mtDNA sequences. As a result, forensic comparisons can be made using a reference sample from any maternal relative, even if the unknown and reference sample are separated by many generations. Anthropologically, mitochondrial DNA in the fossilised source is used to trace the human ancestry particularly of maternal lineage.

Keywords: Mitochondrial DNA; D-loop; Hyper variable region; PCR-RFLP; Sequencing; Hetroplasmy; Maternal lineage.

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Introduction

In Forensic case work analysis, Mitochondrial DNA (mtDNA) is generally used when evidence material contain insufficient amount of DNA. In decomposed or old samples nuclear material in the cell may be less or not exist for a long period, so it is difficult to perform DNA analysis with the nuclear DNA from remains of such biological samples. Mitochondrial DNA has useful genetic information and it degrades slower as compared to nucleic DNA. Mitochondria are present in more number per cell. This high copy number increases the possibility of recovering sufficient DNA from compromised samples, and for this reason, mtDNA can play an important role in the identification of missing persons investigation, in mass disasters, and other forensic investigations involving samples with limited biological material or old decomposed samples. Additionally, mtDNA is maternally inherited [1]. Therefore, barring a mutation, an individual's mother, siblings, as well as all other maternally-related family members will have identical mtDNA sequences. As a result, forensic comparisons can be made using a reference sample from any maternal relative, even if the unknown and reference sample are separated by many generations.

Mitochondrial DNA is transmitted maternally via the egg cell. The human mitochondrial DNA (mtDNA) is a circular double-stranded molecule, and it was first time sequenced by Anderson et al. in 1981 [2]. It has been reported that mtDNA is present in high copy number in human cells, with high mutation rate, the mutation rate is five to ten times higher than that of nuclear DNA. It has three highly variable regions HV1, HV2 and HV3 (non-coding region) which are differentiated by sequencing or by hotspot analysis in which SNPs alleles are identified within these and other regions. Mitochondrial DNA (mtDNA) is a small circular DNA molecule located in mitochondria. Mitochondrial DNA analysis is a valuable technique and its applications are relevant to many different fields [3]. The technique is likely be refined further to provide even more success in the future. In this paper we are reviewing the application possibilities of mtDNA typing in forensic practice.

Structure of Mitochondrial DNA

The mitochondrial genome is circular It has two strands that are differ significantly in their base composition. The heavy strand (H-strand) is purine rich, having a greater number of guanine nucleotides, whereas the light strand (L-strand) is pyrimidine rich and thus physically lighter. The sequence of the mtDNA genome codes for a total of 37 genes, all of which are essential for normal mitochondrial function. A total of 28 gene products are found on the H-strand and 9 on the L-strand. Of the 37 genes, 13 are for proteins which are necessary for cellular respiration, including the NADH dehydrogenase 6 enzyme, 22 are for mitochondrial transfer RNAs (tRNA) and the remaining two encode the 16S and 23S subunits of ribosomal RNA (rRNA) Fig 1 [4]. This types of RNA help to assemble protein building block (amino acid) into functioning proteins.

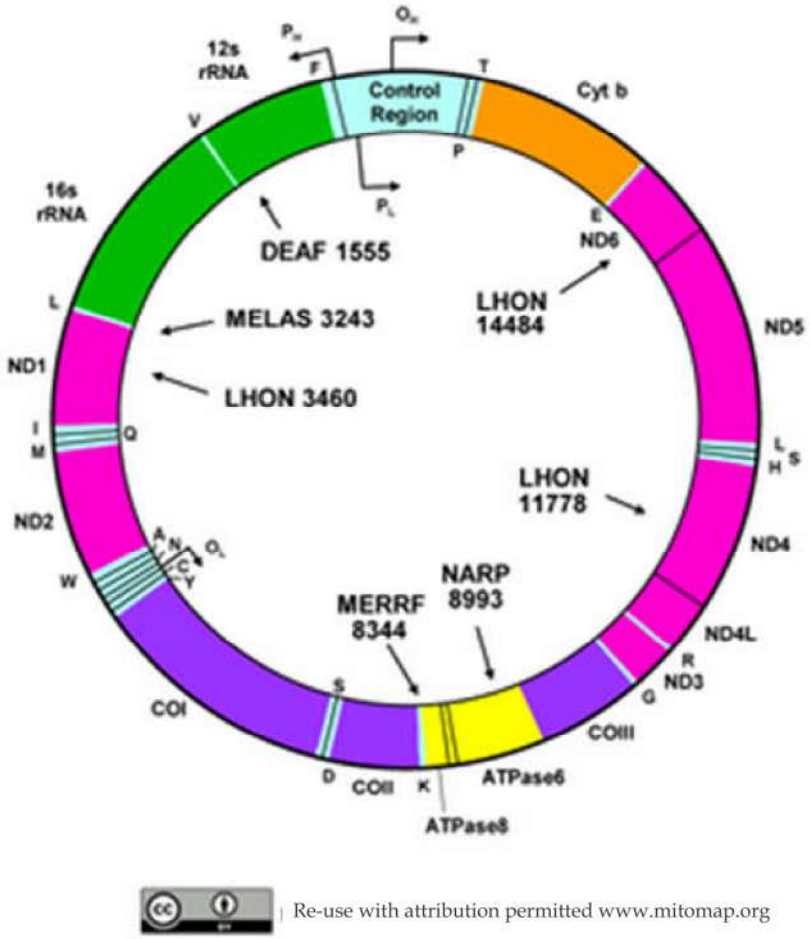

Fig. 1: Structure of Mitochondrial DNA

Configuration of Mitochondrial DNA

The mitochondrial genome is much more efficiently organized than the nuclear DNA genome, containing very little non-coding sequence (7%) compared to the nuclear genome where approximately 97% is not expressed. The 37 genes encoded by the mitochondrial genome are nearly contiguous with each other, lacking introns and only occasionally having one or two base non-coding sequences separating them. Because of this protein coding sequence, there are relatively few sites at which variations in sequence can be tolerated. Therefore, mtDNA is considerably less polymorphic than its nuclear counterpart, despite its higher rate of mutation. In fact, the only significant region of the genome that does not code for a gene product is the displacement loop region, also known as the D-loop. This 1.1 kb stretch of often triplex DNA, known as the "control region", contains essential regulatory functions, including the origin of replication for the generation of multigenic transcripts and DNA replication of the heavy strand. Despite the limited amount of sites for tolerated sequence variations, the mitochondrial genome has been shown to have a mutation rate within the control region up to ten times that of comparable nuclear DNA sequences. Forensic analysts are particularly interested in specific portions of this control region, designated hyper variable regions 1 (HV1) and 2 (HV2), because of their non-regulatory and non-protein-coding status and the dense array of sequence variability they exhibit within human populations [5].

HV1 and HV2 regions span roughly in positions 16024-16383 and 57-372, respectively, whereas the HV3 region spans positions in 438-574 (numbered corresponding to the rCRS (GenBank accession number NC_012920). There are two separate homopolymeric stretches of C nucleotides (poly-C repeats, or C-stretches) in both the HV1 and HV2 regions, therefore there may be possibility of more mutation instead of single nucleotide mutations between individuals. The degrees of polymorphism in the D- loop is so great that direct sequencing may be the most efficient method of typing mtDNA.

MITOMAP, an internet mitochondrial sequence database, maintains a list of published mtDNA polymorphisms found within the D-loop and forensically informative HV1 and HV2 regions (http://mitomap.org/bin/view.pl /MITOMAP/ Polymorphisms Control) [6,7]. Displacement loop or D-loop is a region in the mtDNA structure is consists of a stretch of 1123 base pair sequences. This region is close to the area of mtDNA replication and transcription. It bears two variable regions- HV1 at position 16024-16383 and HV2 at position 57-372.

HV1 Region at position 16023-16383

16023 gttctttcatggggaagcagatttgggtaccacccaagtattgactcacccatcaaca
16081 accgctatgtatttcgtacattactgccagccaccatgaatattgtacggtaccataaat
16141 acttgaccacctgtagtacataaaaacccaatccacatcaaaaccccctccccatgctta
16201 caagcaagtacagcaatcaaccctcaactatcacacatcaactgcaactccaaagccacc
16261 cctcacccactaggataccaacaaacctacccacccttaacagtacatagtacataaagc
16321 catttaccgtacatagcacattacagtcaaatcccttctcgtcccccatggatgacccccc
16383 tca

HV2 at position 57-372

56 atttt
61 cgtctgggggggtatgcacgcgatagcattgcgagacgctggagccggagcaccctatgtc
121 gcagtatctgtctttgattcctgcctcatcctattatttatcgcacctacgttcaatatt
181 acaggcgaacatacttactaaagtgtgttaattaattaatgcttgtaggacataataata
241 acaattgaatgtctgcacagccactttccacacagacatcataacaaaaaatttccacca
301 aacccccctcccccgcttctggccacagcacttaaacacatctctgccaaaccccaaaa
374 acaaagaaccctaa

C-strech region: MtDNAhyper variable region (HV1) contains a C-continuous tract termed the C-stretch, which is located in 16184~16193 nt and is associated with sequence-length variations.This variation have been developed by slipping of the DNA polymerase during replication. The C-stretch evolves much faster than other regions of mtDNA, and variations in this region have been demonstrated widely among unrelated individuals. The mtDNA control region, especially the C-stretch, may be involved in the development of human diseases. It has been reported that C-stretch length heteroplasmy within HV1 and HV2 were observed and the rates of heteroplasmic length variation revealed significant differences among distinct populations. Heteroplasmy is the presence of more than one type of organelles genome within a cell or individual. It is an important factor in considering the severity of mitochondrial diseases because most of the eukaryotic cells contain many hundreds of mitochondria with multiple copies of mitochondrial DNA, it is common for mutation to affect only some mitochondria leaving most unaffected. An individual can exhibit two or more different C-stretch lengths in different tissues as well as in the same tissue, especially in hairs. C-stretch length heteroplasmy was demonstrated in different hair shafts and even in different parts of the same hair. As the 'out-of phase' nucleotide pattern, C-stretch is not easily detected when compared to other regions of mtDNA, and C-stretch length heteroplasmymay be difficult to interpretation of DNA sequencing. C-stretch is located in the middle of mtDNA HV1, and these sequence variations may

hinder the application of the mtDNA control region to forensic and population genetics. Therefore, the C-stretch might be highly significant for forensic identification and population genetic studies [8].

Aspects of Mitochondrial DNA for Forensic purpose

a. Higher Mutation Rate

Mitochondrial DNA has a number of characteristics which makes it an idealchoice for forensic use. It has been estimated that the mtDNA genome evolves at a rate that is up to ten times that of its chromosomal counterpart. Thisis an important factor when considering that data consistently show that unrelated individuals are extremely likely to have different mtDNA haplotypes thus making mtDNA useful for purposes of human identity testing. This higher mutation rate can be accounted for by such factors as DNA repair inefficiencies, oxidative damage, and the greater number of replicate cycles that mtDNA undergoes during cell growth. Evidence also suggests that in spite of such an elevated mutation rate, the majority of mtDNA molecules within a given individual will still be represented by asingle sequence (homoplasmy). Occasionally, however, a *de novo* mutation may occur and propagate, resulting in the phenomenon known as heteroplasmy. Heteroplasmy is a state in which two distinct mtDNA haplotypes coexist within a single individual. This is thought to be due to an mtDNA genome copy "bottleneck" during the early stages of oocyte development. The bottle neck theory purposes that the number of copies of mtDNA in each early oocyte is reduced to a small number of copies as compared to the mature oocyte. Thus, a small number of molecules are chosen as the founder population for all of the mtDNA molecules that are transmitted to the next generation [9]. This set of molecules could contain a homogenous population of mtDNA, orperhaps a heterogeneous mixture due to mutations. Sometimes, such heteroplasmy may increase the discriminatory power of mtDNA identification by providing an additional inclusionary tool for the mitotype, such as situations where an evidentiary sample and a reference sample both exhibit heteroplasmy at the same nucleotide. Other times, it can lead to confusion when comparing two sequences that are assumed to be concordant, as itmay be considered a mixture of mitotypes from more than one individual [10].

b. Inherited Maternally

Human mtDNA is thought to be almost completely maternally inherited. It has been reported that mtDNA is transfer via cytoplasmic inheritance which means via maternal inheritance. This can be explained by the nearly 100,000 copies of the mitochondrial genome residing in the oocyte, and the fact that the few (possibly only two or three) mitochondria present in the spermatozoa are concentrated in the mid-piece and tail region, which are lost following fertilization [11]. Additionally, if the sperm mitochondria do make it to the oocyte, they appear to be preferentially degraded. A group of scientists found a potential explanation. They observed that in *C. elegans* (a type of roundworm), paternal mitochondria are eliminated when the sperm and egg fuse or mt DNA of father (sperm) is eradicated during fertilization. If this process was disturbed, embryo survival rates decreased. This is the first time a study showed experimental evidence to suggest that maintaining paternal mtDNA may be harmful. For this reason it is potentially applicable in identification of maternal relationship. And cannot be used for paternal relationship [12]. Despite this maternal preference, some research has reported a few incidences known as "paternal leakage,"where some paternal inheritance of mtDNA and recombination has occurred. A single case of paternal co-inheritance of mtDNA in humans has been reported so far, in a male individual with a mitochondrial myopathy. In addition, such paternal inheritance of mtDNA has been reported in species ranging from mussels to sheep. Although paternal leakage may occur in rare instances, the normal detectable inheritance pattern of mtDNA is maternal. This maternal inheritance pattern, barring multiple mutations, allows for forensic identifications to be made using reference samples from within the entire maternal lineage, including those that may be separated by several generations, when those of close relatives are no longer obtainable.

c. High copy number

MtDNA is present in a high copy number within most cells. It is estimated that a single cell may contain hundreds of mtDNA genomes for every copy of nuclear DNA. Depending on the needs of the particular cell type, the actual copy number present per cell can vary greatly among different tissue types. For instance, there are more mitochondria in muscle and brain cells than in skin cells. The general abundance of mtDNA can prove vital in situations where the amount of sample may be limited or its quality may be degraded, which is often the case in forensic DNA analyses. Samples that are typical candidates for mtDNA

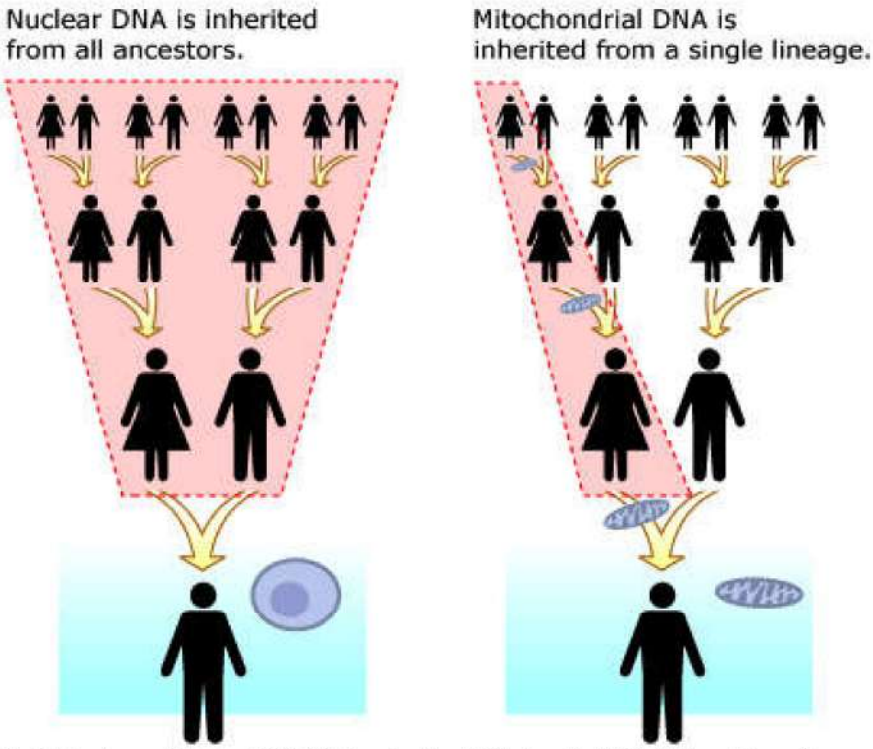

Fig. 2: Inheritance of Nuclear DNA (All Ancestors) and Mitochondrial DNA (Maternal lineage)

analysis include aged bloodstains, skeletal remains, fingernails, teeth, and hair shafts lacking root tissue. The use of mtDNA typing of skeletal remains is often essential in cases of missing persons or in events such as mass disasters where small bone fragments may be the only remaining source of DNA available. In addition, mtDNA testing of hair shafts is of particular importance because shed hairs are common sources of evidentiary material at crime scenes [13].

Molecular Methods for Mitochondrial DNA in Forensic Cases

PCR RFLP: PCR-restriction fragment length polymorphism (RFLP)-based analysis, also known as cleaved amplified polymorphic sequence. It is a popular technique for genetic analysis. It has been applied for the detection of intraspecies as well as interspecies variation. There exist several techniques that are related with PCR-RFLP and also involve gel electrophoresis including techniques for DNA fingerprinting and expression profiling.

PCR-restriction fragment length polymorphism (RFLP)-based analysis is a popular technique for genotyping. The technique exploits that SNPs, MNPs and microindels often are associated with the creation or abolishment of a restriction enzyme recognition site [14]. The first step in a PCR-RFLP analysis is amplification of a fragment containing the variation. This is followed by treatment of the amplified fragment with an appropriate restriction enzyme. Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, allele identification can be done by electrophoretic method in which fragments resolve as per size. Important advantages of the PCR-RFLP technique is that it is not expensive and does not require advanced instruments. The mtDNA D-loop region is useful for PCR RFLP that is amplified by PCR using primers and PCR products can be digested with restriction enzyme such as *Hae* III and *Alu* I. Greater polymorphic rate in the D-loop region has been reported and polymorphism patterns of this region are variable in human population [15]. Disadvantages include the requirement for specific endonucleases and difficulties in identifying the exact variation in the event that several SNPs affect the same restriction enzyme recognition site. Moreover, since PCR-RFLP consists of several steps including an electrophoretic separation i.e relatively time-consuming. This technique is not suitable for the simultaneous analysis of a large

number of different SNPs due to the requirement for a specific primer pair and restriction enzyme for each SNP. This limits its usability for high through put analysis which can be possible by sequencing approach.

Sequencing: In a forensic setting, human mtDNA is analyzed by direct comparison of DNA sequence data of the HV1 and HV2 regions to the rCRS. Standardizing alignments of sequences with the rCRS and following consistent nomenclature for sequence differences is critical to avoid unintentionally describing two sequences as different when in they are actually the same. In fact, several publications have dealt with the nomenclature of sequence data by establishing specific "rules" to follow when determining an mtDNA haplotype. Briefly, differences are reported using the nucleotide positions and the particular base mutation. For example, a sequence that is identical to the rCRS except for having a T instead of a C at position 16150 is designated as 16150T [16].

In the situation of length polymorphisms in the poly-C stretches, any extra Cs are added onto the end of the poly-C stretch. The variant is named using a decimal notation to indicate the number of nucleotides that were in addition to the poly-C repeat in the rCRS. For example, if a particular mtDNA sequence has an additional C compared to the rCRS following the C-stretch of positions 303-315, it would be designated as 315.1C. A similar nomenclature is used to describe insertions or deletions of nucleotides as compared to the rCRS. For instance, if an additional T was inserted following position 294, it would be designated as 294.1T. Finally, deletions are the result of nucleotides that are missing as compared to the rCRS; an mtDNA sequence that was missing nucleotide 325 would be named 325D.

The general rules for naming profiles are as follows as described by Wilson 2002:

- Profiles should be characterized so that the least number of differences from the reference sequence are present.
- If there is more than one way to maintain the same number of differences with respect to the reference sequence, differences should be prioritized as follows:
    1. Insertions/deletions (indels)
    2. Transitions (purine-to-purine or pyrimidine-to-pyrimidine changes)
    3. Trans versions (purine-to-pyrimidine or pyrimidine-to-purine changes)
- As all genes have a 5′ to 3′ direction of transcription and mtDNA genes are encoded on both the heavy and light strands of the closed circular molecule, so that insertions and deletions should be placed 3′ with respect to the light strand of human mtDNA.
- Insertions and deletions should be combined in situations where the same number of differences from the reference sequence is maintained [17].

In order to determine a person's mtDNA haplotype, total genomic DNA is extracted from the biological source material. The extracted DNA is then subjected to amplification of the HV1/ HV2 regions (total of 608bp) using four primer pairs (Table 1). For the HV1 region, two primer pairs, L15997/H16236 and L16159/H16391, are used to amplify overlapping 278 and 271 base pair fragments designated HV1A and HV1B, respectively. The HV2 region is amplified by primer pairs L048/H285 and L172/H408 which typically yields overlapping products of 278 and 277 base pairs designated HV2A and HV2B, respectively (Figure 3). The "L" and "H" designation refers to the light and heavy strand of the mtDNA genome from which the primer sequence is derived and the number indicates the corresponding position of the 3′ end of the primer with respect to the rCRS [18].

The D-loop region of the mitochondrial genome is divided into two main fragments (HV1 and HV2). For universal forensic amplification and sequencing, each fragment is divided into two smaller overlapping fragments (HV1A, HV1B, HV2A and HV2B).

Table 1: Human mtDNA Primers: primers used for control region amplification of human mtDNA.

Hyper variable region 1A (HV1) Primers	
(L15997) A1	5′CAC CAT TAG CAC CCA AAG CT 3′
(H16236) B2	5′ CTT TGG AGT TGC AGT TGA TG 3′
Hyper variable region 1B (HV1) Primers	
(L16159) A2	5′TAT TTG ACC ACC TGT AGT AC 3′
(H16391) B1	5′GAG GAT GGT GGT CAA GGG AC 3′
Hyper variable region 2A (HV2) Primers	
(L48) C1	5′ CTC ACG GGA GCT CTC CAT GC 3′
(H285) D2	5′ GGG GTT TGG TGG AA TTT TTT G 3′
Hyper variable region 2B (HV2) Primers	
(L1720) C2	5′ ATT ATT TAT CGC ACC TAC GT 3′
(H408) D1	5′ CTG TTA AAA GTG CAT ACC GCC A 3′

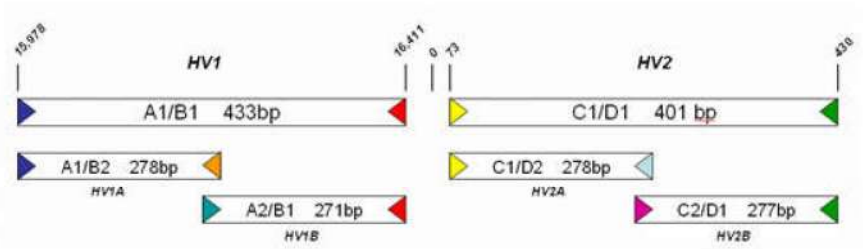

Fig. 3: HV1 and HV2 Primer Overlap Scheme

Sequence analysis

Once the overlapping products are amplified, they are sequenced using the dideoxy chain termination method, *i.e.*, the Sanger method. The Sanger method allows for differential fluorescent labelling of chain terminat or ddNTPs. This allows single reaction sequencing where each label emits fluorescence at a different wave length. In this method, DNA templates are denatured and new strands of DNA are synthesized by *Taq* polymerase. The in corporation of dideoxyribonucleotides creates populations of strands that are terminated with a fluorescent tag at all possible base positions along the template strand. This makes it possible to unambiguously identify the final base of each amplified mtDNA fragment. The resulting sequence product (*i.e.* pool of mtDNA fragments) is then fractionated by capillary electrophoresis (CE) using such commercial systems as the ABI Prism® 310, 3100, or 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). In CE, the terminated DNA chains are subjected to an electric field that separates the amplified fragments based on their size. The amplified products must be separated in order to determine the specific order of incorporated nucleotides across a target sequence. A laser excites the fluorescent dye terminators as they pass a fixed transparent window in the capillary. Light emitted by the excited fluorophores is then detected by a CCD camera. The different bases are ultimately represented as colored peaks on an electropherogram. Next, the data from each individual sequence reaction are parsed to data analysis software, such as Sequencher® (Gene CodesCorp, Ann Arbor, MI) for sequence alignment and examination by an mtDNA analyst [19,20].

Mitochondrial DNA from forensic and clinical samples

Analyses of hyper variable segments of mitochondrial DNA (mtDNA) are used for forensic analysis, human molecular genetics, evolutionary biology, human migration studies and recovery operations in identifying deceased persons, both ancient and modern. mtDNA coding region increases the resolving power of mitochondrial DNA forensic typing.

H.S.P. Garritsen et al. in 2001 studied DNA sequencing to investigate polymorphisms present in two hypervariable segments such as HVR1 and HVR2 (non-coding region) of the mitochondrial genome among 100 platelet apheresis donors. Alignments were made with the Cambridge Reference Sequence (CRS) for human mitochondrial DNA (mtDNA). Combining the sequencing information of HV1 and HV2 they demonstrate that, of the 100 investigated mtDNA samples, none was identical to the CRS. They reported total of 2 ± 17 polymorphisms per donor in the investigated regions, most of them were base pair substitutions (563) and insertions (151). No deletions were found. Sixty-six of the 110 detected polymorphisms were detected in more than one sample. Seven polymorphisms are newly described and have not been published in the Mitomap database. Their results further demonstrated that polymerase chain reaction analysis of the many polymorphisms found in the hypervariable region of mitochondrial DNA represents a more informative target than previously described mitochondrial polymorphisms for discriminating donor ± recipient cells after transfusion or transplantation [21].

A Salas et al. in 2001 reported a forensic case where mtDNA analysis was performed to compare a blood sample obtained from a raped woman with a single hair shaft found in the suspect's car. Two different portions of a single hair shaft were extracted and sequenced for the two non-coding hypervariable segments (HV1 and HV2) of the control region. The results showed differences in sequence between different portions of the hair and the victim's sequence. These differences are related to various heteroplasmy events. The concordance between the hair sample and the potential source (victim) of this sample is questionable and the strength of the evidence depends on how the sequence information is interpreted by the expert. They suggested the necessity to evaluate heteroplasmic events in routine forensic work [22].

Lee H Y et al. 2008 analysed mitochondrial DNA (mtDNA) of control region sequences from highly degraded skeletal remains, they designed seven set of mini-primer, of which four set form HV1 and three set form HV2 region. These modified mini-primer set is less affected by nucleotide variability and was able to amplify the mtDNA sequences of 55-year-old skeletal remains with high efficiency, suggesting that it is a useful tool for analyzing mtDNA control region sequences from highly degraded forensic samples [23].

Mitchell M. Holland et al. 2011 have analysed hypervariable segment 1 (HV1) of the mtDNA control region from 30 individuals using the 454 GS Junior instrument. Mock mixtures were used to evaluate the system's ability to deconvolute mixtures and to reliably detect heteroplasmy, including heteroplasmic differences between 5 family members of the same maternal lineage. Amplicon sequencing was performed on polymerase chain reaction (PCR) products generated with primers that included multiplex identifiers (MID) and adaptors for pyrosequencing. Data analysis was performed using Next *GENe*R software. The analysis of an autosomal short tandem repeat (STR) locus (D18S51) and a Y-STR locus (DYS389 I/II) was simultaneously performed with a portion of HV1 to illustrate that multiplexing can encompass different markers of forensic interest [24].

Doosti A et al. in 2011 studied D-loop region polymorphism in Bakhtiarian population in southwest Iran on 168 healthy people by PCR -RFLP. mtDNA D-loop region was amplified by PCR using specific primers. Restriction fragment length polymorphism (RFLP) was analyzed by *HaeIII* and *AluI* restriction endonuclease. The results of study showed 5 restriction patterns for *HaeIII* enzyme (with 1 heteroplasmy) and 2 restriction patterns for *AluI* enzyme (with 2 heteroplasmies) in Bakhtiarian population. Their findings showed a low level of genetic polymorphism in D-loop region and it is related to high kinship marriages and low range of migration in Bakhtiarian population [25].

Sayed AM et al. in 2017 have conduced study on 36 bone samples from human remains for DNA profiling by Autosomal STRs and Mt DNA polymorphism of control region (D loop) by PCR amplification and sequencing. They reported that MtDNA was more efficient than autosomal STR profiling in discriminating among human bone samples especially those which have low and/or degraded DNA content [26].

Sayed AM Amer et al. 2017 have conducted STR and mitochondrial SNPs technologies for identification of old human bone remains and compared the efficiency of techniques in identifying the bone remains that exposed to severe burning and reported MtDNA was more efficient than autosomal STR profiling in discriminating among human bone samples especially those which have low and/or degraded DNA content [27].

Cai FF et al. in 2011 have studied mutational rate in Mt DNA at D loop region in breast cancer cases. The two hypervariable regions HVR1 and HVR2 in the D-Loop region were sequenced in ten paired tissue and plasma samples from breast cancer patients, they have reported MtDNA mutations were found in all patients' samples, suggesting a 100% detection rate. On examining germline mtDNA mutations, a total of 85 mutations in the D-loop region were found; 31 of these mutations were detected in both tissues and matched plasma samples, the other 54 germline mtDNA mutations were found only in the plasma samples. Regarding somatic mtDNA mutations, a total of 42 mutations in the D-loop region were found in breast cancer tissues. This study concluded that somatic mtDNA mutations in the D-loop region detected in breast cancer tissues were not matched in the plasma samples, suggesting that more sensitive methods will be needed for such detection to be of clinical utility. [28]

Warner JB et al. in 2006 conducted a study on DNA (mtDNA) polymorphisms to detect allogeneic transfused platelets at three hypervariable regions (HVR1, HVR2, and HVR3) within the displacement loop (D-loop) region of the mtDNA, for this PCR sequencing were carried out in 119 unrelated blood donors. The Polymorphic sites were found in all three regions: 66 in HVR1, 44 in HVR2, and 18 in HVR3. All sequence information of HVRs resulted in 105 different genotypes of which 95 were unique. This study showed discriminate between two randomly chosen individuals with a random match probability of 1.2 percent and concluded that D-loop region of mtDNA contains a wealth of informative molecular markers for chimerism and survival studies after transfusions of cellular blood components [29].

Chen F et al. 2009 have conducted study to investigate mitochondrial DNA (mtDNA) hypervariable segment-I (HVS-I) C-stretch variations and explore the significance of these variations in forensic and population genetics studies. The C-stretch sequence variation was studied in 919 unrelated individuals from 8 Chinese ethnic groups using both direct and clone sequencing approaches. Thirty eight C-stretch

haplotypes were identified, and some novel and population specific haplotypes were also detected. The C-stretch genetic diversity (*GD*) values were relatively high, and probability (*P*) values were low. Additionally, C-stretch length heteroplasmy was observed in approximately 9% of individuals studied. There was a significant correlation (r=−0.961, $p<0.01$) between the expansion of the cytosine sequence length in the C-stretch of HVS-I and a reduction in the number of upstream adenines. These results indicate that the C-stretch could be a useful genetic maker in forensic identification of Chinese populations. The results from the Fst and dA genetic distance matrix, neighbour-joining tree, and principal component map also suggest that C-stretch could be used as a reliable genetic marker in population genetics [30].

Senafi S et al. 2014 have conducted the study to amplify HV1 and HV2 regions of human mtDNA to determine individual geographic ancestry using human peripheral blood sample for maternal lineage. Twelve pairs of primers for HV region in human mt DNA were designed after PCR amplification and sequence of six DNA samples were analysed by Mitomap to determine possible haplogroups. Among the analysed samples (1 and 1a, 2 and 2a, 3 and 3a) three haplotypes shown same maternal lineage as they share the same set of mutation in the HV region, they demonstrated that the 12 primer which were designed can be useful for determining haplogroups for geographical ancestry [31].

Ramos A et al. 2013 to investigate the frequency and the mutational spectrum of heteroplasmy in the human mtDNA genome. To address this, a set of nine primer pairs designed to avoid co-amplification of nuclear DNA (nDNA) sequences of mitochondrial origin (NUMTs) was used to amplify the mitochondrial genome in 101 individuals. The analysed individuals represent a collection with a balanced representation of genders and mtDNA haplogroup distribution, similar to that of a Western European population. The results show that the frequency of heteroplasmic individuals exceeds 61%. The frequency of point heteroplasmy is 28.7%, with a wide spread distribution across the entire mtDNA. In addition, an excess of transitions in heteroplasmy were detected, suggesting that genetic driftand/ or selection may be acting to reduce its frequency at population level. In fact, heteroplasmy at highly stable positions might have a greater impact on the viability of mitochondria, suggesting that purifying selection must be operating to prevent their fixation within individuals. This study analyses the frequency of heteroplasmy in a healthy population, carrying out an evolutionary analysis of the detected changes and providing a new perspective with important consequences inmedical, evolutionary and forensic fields [32].

In Indian scenario there are study on simple method of isolation of mtDNA from biological, and a case report on forensic odontology where mtDNA were extracted from human dental tissue samples under prolonged formalin fixation and sequenced (D loop region) successfully. Another study of genetic diversity on non-tribal Indians was conducted to drive maternal lineage by HV1 region by Barnabas S and co-workers at Pune, Maharastra [33, 34, and 35].

Conclusion

The analysis of genetic variation in the nucleotide sequences of mitochondrial DNA has allowed to unravel evolutionary aspects concerning the origin of modern human populations and the clarification of ancient human migration patterns. The differences in mutation rate and heterogeneity between hyper variable regions are enough for individual identification and the maternal lineage of mtDNA. Mitochondrial DNA (mtDNA) analysis has been validated as a useful tool for forensic analysis. Mitochondrial DNA has useful genetic information as they are present in high copy number that increases the possibility of recovering sufficient DNA from compromised samples and for this reason mt DNA is a useful tool in investigation of missing person (unidentified body) by using mt DNA profiling when nuclear DNA STR profiling for Parentage test is not genetically analysed. There are several aspects of techniques for the analysis which need to be considered in order to evaluate the value of the evidence. PCR-RFLP and sequencing generally works well on samples that are unable to be analysed through numerous other techniques. It has been reported that mtDNA analysis may have some times disadvantages in few cases because of low discriminatory power (1:200) and heteroplasmy i.e. few single base pair difference might be there in different cells of the same individual unlike the nuclear DNA.

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