

## Forensic Genetic Analysis of Mitochondrial DNA Hypervariable Region III Sequences in Muslims from South India

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### ABSTRACT

The absence of recombination, high rate of mutation, and maternal inheritance of the mitochondrial DNA (mtDNA) genome makes it a valuable tool in many fields including evolutionary anthropology, genetic genealogy, population history, and forensic science. The mtDNA genome can be separated into two parts: a large coding region and a smaller fragment called the control region or non-coding region that generally referred to as displacement loop (D loop). The mtDNA D-loop region was highly polymorphic and has proven a precious marker in forensic identification. The study aims to examine and suggests polymorphism of the HVRIII region as a power marker along with HVRI and HVRII in forensic investigations. Within the control region of the mtDNA genome, the sequences of hypervariable region III (HVR III) (nucleotide position 438-574) were obtained from 60 unrelated Muslims of Shrirangapattana town, located in Karnataka state of South India. The complete mtDNA control region was amplified and sequenced by the Sanger sequencing method. The study provided the identification of 18 different haplotypes and 17 polymorphic nucleotide positions. The most common haplotype (H.18) was consistent with the Anderson sequence which occurred fourteen times. The distribution of nucleotide substitutions, insertions, and deletions was computed and determined that transitions made up the majority of the variations (58%) in this region. The genetic diversity was estimated at 0.89939 and the random match probability at 0.1155. The power of discrimination was found to be 0.8844 and the rest of the statistical parameters such as the mean of pair-wise differences and nucleotide diversity were established as  $2.255932 \pm 1.25884$  and  $0.010071 \pm 0.00623$ , respectively. Consequently, the discovery of high genetic, haplotype, and nucleotide diversity, and high power of discrimination impart the use of hypervariable region III (HVR III) as an important marker in forensic investigations.

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### Introduction

The mtDNA sequence analysis in the D-loop region has found a useful tool in forensic investigations and matrilineal origin research, especially when there is insufficient nuclear DNA in samples for typing (Hwa *et al.*, 2010; Panneerchelvam *et al.*, 2010). The absence of recombination, high copy number, and circular form of mtDNA make it a valuable marker in

identifying persons from the highly degraded biological sample in the human identification field (Fridman and Gonzalez, 2009). Although the most extensive mtDNA variations between individuals are found within the two segments of control region hypervariable region I (HVRI) (np 16024 to 16365) and hypervariable region II (HVRII) (np 73 to 340), occasionally a third portion of the control region, known as



hypervariable region III (HVRIII) (np 438 to 574) can provide evidence with the identity of crime victims (Bini *et al.*, 2003; Mabuchi *et al.*, 2007) especially when sequences of HVRI and HVRII are identical, the additional polymorphic site within HVRIII can be helpful to identification such indistinguishable HVRI/HVRII samples (Baasner *et al.*, 1998; Lutz *et al.*, 2000; Budowle *et al.*, 2003). Different investigations have been shown the potential value of discrimination and identification using polymorphic sites in HVRI and HVRII regions (Imaizumi *et al.*, 2002; Bini *et al.*, 2003; Gabriel *et al.*, 2003; Divne *et al.*, 2005; Tsutsumi *et al.*, 2006; Mabuchi *et al.*, 2007; Lander *et al.*, 2008; Lehocký *et al.*, 2008; Fridman and Gonzalez, 2009); however, only a few groups have been working with HVRIII analysis for this purpose (Andrews *et al.*, 1999; Lutz *et al.*, 2000; Nagai *et al.*, 2004; Lee *et al.*, 2006; Hameed and Jebor, 2016). In this study, we evaluated the variability of the HVRIII region in the Indian Muslim population to find polymorphic positions that fulfill the situations necessary for their future application in the identification process and forensic investigation casework.

## Materials and methods

### Population and DNA extraction

After obtaining written consent, which was approved by the ethical committee of the University of Mysore, blood samples were obtained from 60 randomly chosen unrelated Indian Muslim individuals of Shirangapattana. From each 5ml of intravenous blood was collected in 6 ml EDTA-K2 tubes (BD Vacutainer). The blood samples were subjected to DNA extraction using a DNA extraction kit, QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. From the extracted DNA the targeted mtDNA region was amplified using the polymerase chain reaction (PCR) technique with the help of specified primers and sequences obtained by using Sanger's method. Mutations of these sequences were scored after comparing with the Revised Cambridge Reference Sequence and further analyzed by using an appropriate statistical tool.

### PCR amplification and sequencing

For PCR amplification of the HVRIII region of mtDNA, the following primers were used: HV3F (5'-GAGCCCGTCTAAACATTTTCAG) and HV3R (5'-CAGCACTTAAACACATCTCTGC). PCR amplification was carried out in 40 µl reaction mixture subjected to 35 cycles of 95 °C for 3 min, 95 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. The DNA sequencing was carried out in Agrigenome labs (A subsidiary of Scigenom labs) using HV3F forward and HV3R reverse primers by Sanger's method.

### Statistical Analysis

The sequences of HVRIII (position 438-574) aligned and compared with the Revised Cambridge Reference Sequence (rCRS) (Andrews *et al.*, 1999) using clustal W software in the bioEdit version 7.2.6. The probability of randomly selecting two individuals with the same mitochondrial profile [random match probability ( $p$ )] was defined by an equation  $P = \sum X^2$ , where X is the frequency of each observed mtDNA haplotype (Stoneking *et al.*, 1991). The genetic diversity ( $h$ ) was calculated following  $h = n(1 - \sum X^2) / (n-1)$ , where n is the sample size and X is the frequency for each observed haplotype, and The discrimination power (DP) was also estimated using the equation  $DP = (1 - \sum X^2)$ , where X is the frequency of each observed haplotype (Tajima, 1989). The rest of the statistical parameters were calculated using Arlequin version 3.5.2 software (Excoffier and Lischer, 2010).

### Results and discussion

After performance sequence alignment against the Revised Cambridge Reference Sequence, variations from the rCRS represented mutations, nucleotide positions of these mutations were determined and subsequently, mitochondrial haplotypes were concluded and correlated to the published databases. To investigate the HVRIII sequences from nucleotide position 438 to 574, the data were categorized into nucleotide substitutions, deletions, and insertions. Seventeen different variable sites of HVRIII were observed, as shown in table 1.

The most frequently observed nucleotide substitution was at position 489 (T to C) and secondly at position 523 (A deletion) and 524 (C deletion). T to C transition at np 489 is the most frequent variant in the human population. In the present study, it is observed to be 63.34 percent. Similar high polymorphism at np 489 found in Thai populations (66.67%) (Thongngam *et al.*, 2016), Japanese (64.67%) (Nagai *et al.*, 2004) and (64.3%) (Tsustumi *et al.*, 2006), Koreans (61.82%) (Zhang *et al.*, 2005), Han Chinese (56.86) (Zhang YJ *et al.*, 2005), Hong Kong people (50.93%) (Irwin *et al.*, 2009), and Malaysians (45.16%) (Nur Haslindawaty *et al.*, 2010), while T489C nucleotide variation in Iraqis (4.8%) (Shneewer *et al.*, 2015), Venezuelans (29%) (Lander *et al.*, 2008), and Germans (6.5%) (Lutz *et al.*, 1998) showed lower polymorphism. This may be region-specific to Southeast Asian populations. C to G

at np 447 characterizes haplogroup M2. M2 haplogroup is specific to Indian (Chandrasekar *et al.*, 2009). In this study, it forms the H3 haplotype with a frequency of about 12 percent. Transition C to T at np 461 is specific to the M6 haplogroup and Southeast Asia, particularly the Indian subcontinent. The next variant at np 462 is more frequent in the mtDNA database and it is nested with J1b, J1c, and J1d haplogroup trees. It probably originated in the near east and frequent in Pakistan (Quintana *et al.*, 2004). Transition T-C at np 480 is frequent in European lineages whereas the same at np 482 is frequent in Indian populations. G499A is about 6 percent in the mitomap database and frequent in the American continent (B2 haplogroup). Haplotype H12, A508G nested with U2e lineage which is frequent in Italy (Achilli *et al.*, 2007). In the present study, it forms haplotype H6.

**Table 1.** Sequence polymorphisms in the mtDNA HVRIII region of 60 unrelated Indian Muslims.

Anderson	447C	461C	462C	480T	482T	489T	499G	508A	511C	523A	524C	524a*	524b*	524c*	524d*	573.1	574A	Number of Individual
H*1	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	2
H2	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	9
H3	G	.	.	.	.	C	.	.	.	D	D	.	.	.	.	.	.	7
H4	.	.	T	.	.	C	.	.	.	.	.	.	.	.	.	.	.	5
H5	.	.	.	.	.	C	.	.	.	D	D	.	.	.	.	.	.	2
H6	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	1
H7	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	1
H8	.	.	.	.	.	.	.	.	.	D	D	.	.	.	.	.	.	4
H9	.	.	.	.	C	C	.	.	.	D	D	.	.	.	.	.	.	2
H10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	1
H11	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	5
H12	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	1
H13	.	.	.	.	.	C	.	.	.	.	.	A	C	.	.	.	.	1
H14	.	.	.	.	.	C	.	.	.	.	.	A	C	A	C	.	.	1
H15	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	1
H16	.	T	.	.	.	C	.	.	.	D	D	.	.	.	.	.	.	2
H17	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	C	.	1
H18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	14
Total	.	.	.	.	.	12	.	.	.	.	.	.	.	.	.	.	.	60

H\*: haplotype; G: Guanine; T: Thiamine; C: Cytosine; A: Adenine; D: deletion; (a-d)\*: indicate additions of nucleotides not found in the reference sequence.

From HVRIII region of mtDNA, 18 different haplotypes were identified (Table 2). Among them, 8 types were observed only once and the other 10 haplotypes were shared between multiple individuals; 4 types were observed twice. The haplotype no.18 was the most frequently detected haplotype, which is the reference sequence (rCRS). Reported variable sequences in this manuscript are deposited in the Genbank under accession number MK104528-MK104587.

**Table 2.** mtDNA HVRIII haplotypes among the 60 unrelated Indian Muslims.

No	HVRIII-haplotype (438-574)	Frequency	Percentage
H*1	489, 511	2	0.0333
H2	489	9	0.1500
H3	447, 489, 523 D, 524 D	7	0.1167
H4	462, 489	5	0.0833
H5	489, 523 D, 524 D	2	0.0333
H6	508	1	0.0167
H7	523 D, 524 D	4	0.0667
H8	482, 489	5	0.0833
H9	499, 574	1	0.0167
H10	489, 524.1 A, 524.1 C	1	0.0167
H11	489, 524.2 A, 524.2 C	1	0.0167
H12	482, 489, 523 D, 524 D	2	0.0333
H13	480	1	0.0167
H14	461, 489, 523 D, 524 D	2	0.0333
H15	489, 574	1	0.0167
H16	482, 489, 573.1 C	1	0.0167
H17	573.1 C	1	0.0167
H18	rCRS	14	0.2333
	Total	60	1

H\*: haplotype; D: deletion; 0.1, 0.2: Insertion; rCRS: Revised Cambridge Reference Sequence.

Table 3 exhibits the frequency distribution of nucleotide substitutions, insertions, and deletions. It was observed that transitions (58%) were more prevalent than transversions (14%). It is also observed that deletions were more than insertions in the HVRIII region of mtDNA. Nevertheless, the HVR III has proven to be useful as a supplementary polymorphic segment for forensic application (Hoong and Lek, 2005). Even though the majority of forensic cases utilize only the HVR I and HVR II sequences, it is noteworthy that HVR III (AC)<sub>n</sub> repeat segment has equal potential as an additional

mtDNA marker in forensic investigations (Ivanov *et al.*, 1996; Lutz *et al.*, 2000; Chung *et al.*, 2005; Fridman and Gonzalez, 2009; Bhatti *et al.*, 2018). The nucleotide insertion was observed in the present study, at two positions with +C at position 573, +AC at position 524, and + ACAC at position 524. Nucleotide deletion was also observed at nucleotide position 522-523 was a deletion of -(AC), which was found in 5 of 18 individuals in our study. (CA)<sub>n</sub> dinucleotide indels of mtDNA HVR III 514-524 region in the Indian population has been found in the Urali Kuruman tribe of Kerala, South India. Among the Uralikuruman, CA<sub>4</sub> is the common one. (Sylvester *et al.*, 2018). It was also found that CA<sub>4</sub> was common among the Thai population (Thongngam *et al.*, 2016) whereas the common CA repeat observation in the present studied population was CA<sub>5</sub>.

**Table 3.** Nucleotide substitutions, insertions, and deletions in the mitochondrial DNA hypervariable HVRIII region of 60 unrelated Indian Muslims.

Mutation type	Number of positions	Total number of mutations
Transition		
C → T	3	9
T → C	3	39
A → G	1	1
G → A	1	1
Total	8	50
Transversion		
C → G	1	7
A → C	1	2
Total	2	9
Insertion		
+1AC (at np 524)	1	1
+2AC (at np 524)	1	1
+C	1	2
Total	3	4
Deletion		
-AC	1	17
Total	1	17

Diversity parameters for the HVRIII region of mtDNA in Indian Muslims of the present study are given in Table 4. A statistical estimate of the results for this population showed a genetic diversity (h) of 0.89939 and it was calculated between 0.6 to 0.8 by several authors (Nagai *et al.*, 2004; Vanecek *et al.*, 2004; Lee *et al.*, 2006). The probability of random match (p) of two individuals showing the same mtDNA

haplotype was 0.1155 and it was defined between the ranges of 0.2 to 0.4 in different human population studies (Bini *et al.*, 2003; Nagai *et al.*, 2004; Vanecek *et al.*, 2004; Lee *et al.*, 2006; Palencia *et al.*, 2010). Similar to the present work high genetic diversity (h) 0.860 for the HVR-III region and the power (1-p) of discrimination 0.846 for HVR-III among the Tai populations was found (Chen *et al.*, 2002).

In this study, the power of calculated discrimination was 0.8844, and statistical parameters such as nucleotide diversity and mean the number of pair-wise differences was estimated as  $0.010071 \pm 0.006230$  and  $2.255932 \pm 10258400$  respectively. Thus, the high genetic diversity for the HVRIII region in the present studied population was found.

**Table 4.** Diversity measures for 60 unrelated Indian Muslims.

Parameters	HVIII
Genetic diversity (h)	0.89939
Random match probability (P)	0.1155
Nucleotide diversity ( $\pi_n$ )	$0.010071 \pm 0.006230$
Mean number of pairwise differences ( $\pi$ )	$2.255932 \pm 1.258400$
Power of discrimination (PD)	0.8844

## Conclusion

Sequence databases are the best source of information regarding the power of mtDNA for identity testing. We have examined the nucleotide diversity of the HVRIII region in 60 unrelated individuals of a socially and culturally distinct group. A total of seventeen variations were observed in the 137 bp length stretch of the HVRIII region. The nucleotide transitions are found to be more predominant than the transversions (50:9). T to C transition was found to be the most frequent substitution (66%). The present studied population has CA<sub>5</sub> type of CA repeat as the most common type. It reveals that the present population has more genetic relation with European populations. The high diversity (0.8994), power of discrimination (0.8844) for the HVRIII region of the studied population is in agreement with the results of similar studies. Although combined HVRI, HVRII, and HVRIII data (D loop) together by having more variable site and more haplotypes increase the power of discrimination, identification, and the genetic diversity of any population but the additional

polymorphic sites within the HVRIII region help to resolve indistinguishable HVRI/HVRII samples and can be used as an additional marker in genetic differentiation of human populations and forensic investigation casework.

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## Conflicts of interest

The authors declare that they have no conflict of interest.

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