# Mitochondrial DNA Markers of Hair Shaft in Iraqi population

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***Abstract***

*Mitochondrial DNA control region from scalp hair shafts was collected from 100 random healthy unrelated individuals of three generations who were representative of the Arab Iraqi population. This study aimed to detect the mtDNA variation markers that are useful for forensic identification and generating a baseline genetic structure of the Iraqi population. In order to estimate the amount of mtDNA in a hair shaft, SYBR Green Real-time PCR quantification was used. The mtDNA variations within the control region were analyzed by Sanger DNA sequencing that showed 162 polymorphic nucleotide positions involved in the composition of different haplotypes and classification into the haplogroups H (26%), U (14%), HV (11%), J (10%), T (10%), R (8%), L (7%), K (5%), N (4%), B (3%), A (1%) and I (1%).*

*Principal component analysis revealed the relationship between Iraqi and Saudi, Jordanian, and Iranian populations, although the Iraqi mtDNA composition was heterogeneous. The genetic diversity, which was 99.75%, was significant and reflected a probability of two randomly selected individuals from a population owning an identical mtDNA haplotype as being 0.0291.*

**Keywords:** Mitochondrial DNA, Control region, Forensic markers, Iraq.

## Introduction

A hair can provide evidence in a forensic laboratory and may be a probative clue because the hair is very stable. A single head hair can provide as much information about an individual as continuously shedding human head hairs. The hair shaft is the most credible forensic evidence because the hair shaft shed can be subjected to mitochondrial DNA (mtDNA) when mtDNA copies are available[1].

The hair shaft is hard because it contains mostly keratin[2]; the mtDNA molecules are composed of a compact structure called the nucleoid, which is in turn covered with a protein shell, which protects the mtDNA molecules. The nucleoid may be resistant to digestion by nucleases[3].

The analysis of the control region of mtDNA is commonly used for forensic identification of non-nuclear DNA; in addition, there is a large, global mtDNA database of hypervariable regions of mtDNA control region available with universal primers that can be used to obtain an output for any unknown sample and to discriminate between unrelated individuals[4].

In this study, we have used the mtDNA haplogroup analysis which has been widely used in various disciplines including studies of population genetics of Iraqis[5,6]. In our study, all hair samples were typed for mtDNA of Arab ethnicity because the Iraqi population is predominantly composed of Arabs (75–80%). Iraq is located in the Middle East and bordered by Arab countries including Saudi Arabia, Syria, Jordan and Kuwait, but not Turkey and Iran.

## Material and Methods

**Sampling and DNA Extraction of Hair Shaft:** Scalp hairs of 100 unrelated individuals of three consecutive generations were collected from different regions of south Iraq. All participants signed an informed consent form prior to participation. This study was approved by the Scientific Committee of the Biology Department, College of Science, Al Muthanna University. The pretreatment protocol consisted of washing of the hair samples with 500 µl of 70% ethanol and then $H_2O$. The hair samples were examined further under a magnifying glass to ensure that there was no contamination and then left to air-dry. The genomic DNA was extracted from the hair shafts by using biotrace purification kit (EURX, Poland).

**Mitochondrial DNA Copy Number:** The copy number of mtDNA was estimated by SYBR Green Real-time PCR analysis (Agilent Mx3000p, USA) of mitochondrial genes, NADH dehydrogenase subunit 1 (ND1) and NADH dehydrogenase subunit 5 (ND5). Total cellular DNA was collected and amplified. ND1 and ND5 copy number were normalized to half the level of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) since each cell contains two copies of genomic DNA compared to a single copy of DNA per chromosome. The primers of mitochondrial ND1 and ND5 genes and nuclear GAPDH gene were described in the previous study by Yu et al[7] as shown in table 1.

The qPCR components were 25 µl of 2X Go*Taq* qPCR master mix with 0.4 µl of Carboxy-X-Rhodamine (CXR) reference dye (Promega, USA), 1µl of 10 µM primer mix (forward and reverse), 2 µl of genomic DNA of 2 cm of hair shaft sample and completed to a final volume of 40 µl. The real-time PCR conditions were as follows cycle 1 at 95°C for 30 seconds, then 40 cycles at 95°C for 5 seconds each and annealing/extension at 60°C for 30 seconds. Subsequently, a melting curve analysis at dissociation temperature of 60–95°C was performed.

Data Analysis was performed, CT values of mtDNA and nuclear DNA (nuDNA) were obtained from the real-time PCR software and the average of the CT values was taken from triplicate reactions. The mtDNA content relative to nuDNA was determined using the following equations[8]:

(a) $\Delta CT = (nuDNA\ CT - mtDNA\ CT)$

(b) Relative mitochondrial DNA content $= 2 \times 2^{\Delta CT}$

**Amplification of Mitochondrial DNA:** The amplification of three overlapping segments of mtDNA control region extending from 15995-16488, 16221-259 and 182-619 nucleotide positions was done according to the revised Cambridge Reference Sequence[9] using L15995, H16488, L16221, H259, L182 and H619[10] as listed in table 1. PCR components included 25 µl One*Taq* Quick-Load 2X Master Mix with standard buffer, 1 µl of primer mix (10 µM), 3 µl of template DNA and nuclease-free water completed to a final volume of 50 µl. The PCR conditions were as follows: 1 cycle of initial denaturation at 94°C for 4 minutes and 40 cycle of denaturation at 94°C for 30 seconds, annealing at 54°C for 1 minute and extension at 68°C for 1 minute, then 1cycle of final extension at 68°C for 5 minutes.

The amplified products were subjected to direct DNA sequencing by using ABI 3730 XL DNA analyzer (Applied Biosystem, USA) using universal (M13) and reverse sequencing primers as shown in table 1[10]. The DNA sequence data were analyzed by using BioEdit alignment editor Software. The distribution of mtDNA haplogroups was ascertained using mtDNA manager that enables automatic estimation of the mtDNA haplogroups according to control region variations[11].

**Statistical Analysis:** The statistical analysis of the quantitation of mtDNA was performed by using the t-test. The frequency of Iraqi mtDNA haplogroups is related to several populations around the world. The statistical analysis of the genetic diversity and random match probability were calculated according to the Tajima[12] study of polymorphisms within the mtDNA of the Iraqi population.

## Results and Discussion

**Quantitation of mtDNA to nuDNA by Real –Time PCR:** The quantification of mtDNA: nuDNA ratio by SYBR Green real-time PCR in this study was carried out to provide information about the amount of genetic material present in hair shaft samples. Mitochondrial genome of hair samples can be used for identification purposes in humans because a greater amount of as compared to nuDNA is found in hair shafts. nuDNA is useful where the hair shaft has not been cut or damaged[13]. The relative quantitation of mtDNA in 100 hair shaft samples revealed that the copy number ranged 19-74941 copies per 2cm of hair shaft (average= 3557; S.D.=9945). The rate and range variations of mtDNA content in the hair shaft of different individuals are dependent on the hair type, whether the hair is shed or plucked and the distance from the root[14]. There was no significant difference ($P < 0.05$) in mtDNA copy number between males and females table 2. The study by Xia et al[15] reported that the mtDNA content of blood samples showed no differences between the genders.

**Table 1**
**The primers used in this study that include real-time PCR primers for estimation of copy number of mitochondrial DNA (mtDNA), primers of conventional PCR for amplification of control region in mtDNA and DNA sequencing primers of PCR products.**

| Primer of Real-time PCR | Sequence- 5´ -- 3´ | | Product size (bp) |
|---|---|---|---|
| ND1 | Forward | TTCTAATCGCAATGGCATTCCT | 109 |
| | Reverse | AAGGGTTGTAGTAGCCCGTAG | |
| ND5 | Forward | TTCATCCCTGTAGCATTGTTCG | 154 |
| | Reverse | TTCATCCCTGTAGCATTGTTCG | |
| GAPDH | Forward | CAGAACATCATCCCTGCCTCTAC | 251 |
| | Reverse | TTGAAGTCAGAGGAGACCACCTG | |
| **Primers of conventional PCR** | **Sequence5´ -- 3´** | | **Product size (bp)** |
| L15995 | CGTAAAACGACGGCCAGTGAACTCCACCATTAGCACCCAAAG | | 516 |
| H1648 | GGAAACAGCTATGACCATGAGGAACCAGATGTCGGATACAG | | |
| L16221 | GTAAAACGACGGCCAGTGAACAAGCAAGTACAGCAATCAAC | | 575 |
| H259 | GGAAACAGCTATGACCATGGATGTCTGTGTGGAAAGTGGCT | | |
| L182 | ´GTAAAACGACGGCCAGTGACGCACCTACGTTCAATATTAC | | 688 |
| H619 | CGGAAACAGCTATGACCATGGGTGATGTGAGCCCCGTCTAA | | |
| **Sequencing primer** | **Sequence 5´ -- 3´** | | |
| Universal (M13) | GTAAAACGACGGCCAGTGA | | |
| Reverse | GGAAACAGCTATGACCATG | | |

**Mitochondrial DNA Haplogroup:** The mtDNA sequencing results showed that among the 162 polymorphic nucleotide positions within the control region of the Arab Iraqi population, the frequencies of the haplogroups using the mtDNA manger software were as follows: H 26 %, U 14%, HV 11%, T 10%, J 10%, R 8%, L 7%, K 5%, N 4%, B 3% and A 1%. Of the previous studies conducted on the control region of mtDNA in the Iraqi population, the Al-Zahery et al[16] study reported the following haplogroups: Pre-HV (4.2%), HV (10.6%) and H (19.9%). Basheer et al[17] study reported the following haplogroups: HV (6.4%) and H (16.9%). The frequencies of the haplogroups Pre-HV, HV and H in the Iraqi population are comparable to that of Jordanians[18], Saudis[19] and Iranians[5].

The frequencies of Jordanians and Saudis are close to Iraqis followed by Iran. The haplogroups H and HV are important determinants of Middle Eastern origin, although the frequency of haplogroup H in Europe is high (35-50%), but HV is higher in Middle East on H about (15-30%) , that lends support to the hypothesis that haplogroup H originated in the Middle East[6]. The frequency of mtDNA haplogroups U was 14% (the frequency of sub-clade U5 was 9%) in this study; haplogroup U is close to Europe[20] and it causes little effect to separate Iraq from other Arabic and Middle East countries and close to Saudi and Iranian. The frequency of mtDNA haplogroups J was 10%.

Previous studies in the Iraqi population have demonstrated a frequency 9.3% and 8.6%, which is less than that in Saudis (19.4%) and Iranians (13.5%). The frequency of haplogroups L in the Iraqi population was observed to be 7% in the present study that may reflect the gene flow from African population. Haplogroup L is regarded as a macrohaplogroup and the root of the mitochondrial phylogenetic tree; it also represents the common African mitochondrial origin of entire mankind[21]. A total frequency of 6% was recorded for the haplogroups B. K, W, X and I, these haplogroups are rare in the Iraqi population. The lack of the haplogroup V in the Iraqi population suggests that the gene flow is undoubtedly from the European gene pool reported by some authors[17,23].

The study of the Arab Iraqi population by the principal component (PC) analysis of mtDNA control region is shown in figure 1. The statistical analysis of the Iraqi population was found to display the large heterogeneity. It is difficult to define the migration line for Iraqi society. PC analysis relied on Pre-HV, H, U and R haplogroups for Iraq, Jordan and Iran on HV, H and U haplogroups for Iraq and Saudi Arab in the same component.

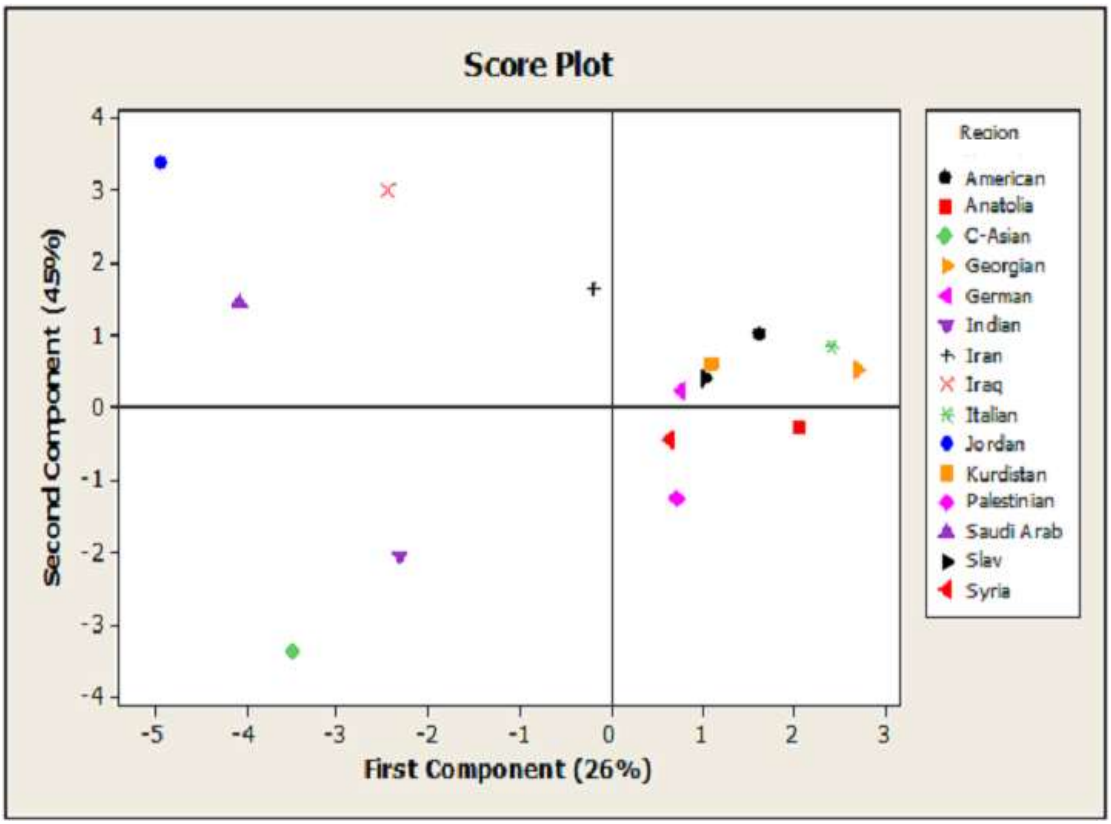


**Figure 1: Principal Component analysis of the mtDNA haplogroups. Each population under study is depicted in a different color shade and shape. On the whole, 71% of the total variance is represented: 26% by the first component and 45% by the second component. References of data: Iranian[5], Slavic[5], Indian[5], Syrian[6], Palestinian[6], German[10], Jordanian[18], Saudi[19], Kurdistan[22], Italian[23], American[24], Anatolia[24], Georgian[24] and C-Asian[25].**

**Table 2**
**Results of two-sample t-test for copy number vs. gender**.

| Gender | n | Mean | St Dev | SE Mean |
|---|---|---|---|---|
| Female | 54 | 3559 | 10791 | 1468 |
| Male | 46 | 3555 | 8971 | 1323 |

Abbreviations: n=Total number of subjects; SE Mean= Standard error of mean; St Dev= Standard deviation

The PC analysis separated the genetic unit flow from populations of Turkish origin (Anatolia) that included Syrians and Palestinians with the Anatolian population in one group; the results underscore the hypothesis of the migration from the Levant countries and compromised in the European gene pool because Anatolia has served as a demarcation line between the Middle East and Europe. The first component included Anatolia (Palestine and Syria). The second component included Iraq with Saudi Arabia, Jordian and Iranian populations and distance from other Arabic and Middle Eastern countries i.e. Iraq, Jordan, Iran and Saudi Arabia.

Study of the Iraqi population showed absence of haplogroups D and M, specific haplogroups of gene flow from India and Central Asia. The statistical analysis of these population groups revealed a large heterogeneity. The PC analysis relied in all likelihood, on H, J, T, K, B, N and T haplogroups.

**Genetic Diversity and Random Match Probability:** The main parameters for statistical evaluation of mtDNA analysis are based on the random match probability and genetic diversity in human identification. Our study showed that the genetic diversity was 99.75% that was reflected in the probability of two randomly selected persons from a population owing identical mtDNA haplotypes as being 0.0291.

## Conclusion

The relative quantitation of mtDNA of hair shaft samples revealed a wide range of copy number values per 2 cm of hair shaft. The analysis of mtDNA copy number showed that there is no significant difference between males and females. The mtDNA sequencing detected 162 polymorphic nucleotide positions within the control region and among the different haplogroups of the Arab Iraqi population. The PC analysis showed that the Iraqi population shared one with Jordan, Arab Saudi and Iran.

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