ANTHROPOLOGIE • XLVII/1–2 • pp. 107–118 • 2009

SABEH FRIGI, HAJER ENNAFAA, LOTFI CHERNI, AMEL EL GAAIED

INVESTIGATION OF POLYMORPHISMS IN HYPERVARIABLE REGION II (HVII) SEQUENCES OF HUMAN MITOCHONDRIAL DNA IN TWO NORTH-AFRICAN POPULATIONS

ABSTRACT: MtDNA analysis has been focused mainly on the first hypervariable region (HVI). This paper deals with polymorphisms in the HVII of two Tunisian populations (Sejnane and Takrouna). Particular attention was paid to nucleotide site 73 which is particularly informative when studying European populations as well as polymorphisms in a mononucleotide repeat poly-C stretch between position 303 and 315 nucleotides (D310) which has been identified recently as a frequent hot-spot of deletion/insertion mutations in tumors. Our results demonstrate that site 73 plays a central role in distinguishing between haplogroups. We show that the subset of sequences carrying adenine at site 73 belongs to haplogroups H and V and has a much more recent common mitochondrial DNA ancestor than the subset of sequences with guanine at that site. The polymorphism of the 303-315 mitochondrial microsatellite in the two communities shows that this mitochondrial C-stretch is a frequent hot spot of mononucleotide insertions. The results showed that the two haplotypes 309.1 315.1 and 309-315.1 are the most frequent in all studied populations. Our results also confirm the high diversity of the population of Sejnane compared with Takrouna and other populations.

KEY WORDS: Mitochondrial DNA – Polymorphism – HVII – Site 73 – Microsatellite 303-315 – Tunisian Berbers

INTRODUCTION

Since the publication of the complete sequence of the human mitochondrial genome, mitochondrial DNA (mtDNA) has been used in forensic identity testing (Richards *et al.* 2000) and especially in human population studies (Watson *et al.* 1997, Cerny *et al.* 2008, Coudray *et al.* 2009). For these purposes mtDNA is particularly useful due to its unique properties, such as high number of copies per cell, maternal inheritance, lack of recombination and fast substitution rate. Differences between mtDNA sequences are only due to mutation. As time passes, mutations accumulate sequentially along less and less related molecules that constitute independent lineages known as haplotypes. Relationships among lineages can be estimated by phylogenetic networks (Bandelt *et al*. 1995) where mutations are classified at hierarchical levels. Basal mutations are shared by clusters of lineages, defined as haplogroups, whereas those at the tips characterize individuals. Major haplogroups are continental or ethnically specific (Richards *et al.* 2000).

Within the mitochondrial genome, the most variable region is the control region and within it the most polymorphic nucleotide sites are concentrated in two hypervariable regions. The large majority of mtDNA sequence data published to date is limited to the most variable of these two regions: hypervariable region I. In the present paper we concentrate on the polymorphisms of hypervariable region II.

Our purpose is first to evaluate the phylogenetic importance of a specific nucleotide in hypervariable region II, site 73 (numbering after Anderson *et al.* 1981), which is particularly informative when studying European populations (Wilkinsons *et al.* 1996). Secondly we studied the polymorphism of the poly-C tracts of hypervariable region II

(located between positions 303 and 315), which is known to have a high insertion/deletion rate, thus originating length heteroplasmy (Hauswirth, Clayton 1985). This type of heteroplasmy is represented by multiple populations of mtDNA of a poly-C stretches of various lengths (Lee *et al.* 2004). In fact, this mononucleotide repeat has been identified recently as a frequent hot-spot of deletion/insertion mutations in tumors (Sanchez-Cespedes *et al.* 2001). This homopolymeric C-stretch (CCCCCCCTCCCCC) is part of the conserved sequence block II located within the regulatory D-loop region and involved in the formation of a persistent RNA-DNA hybrid that leads to the initiation of mtDNA heavy-strand replication (Lee *et al.* 1998).

MATERIAL AND METHODS

Populations

Takrouna is a small village close to the littoral town of Sousse that crowns the mountain Takrouna, at a height of approximately 230 m. It is surrounded by two other Berber villages: Zriba and Jeradou, with which it had historical and ancient relationships. It covers 320 hectares and has a population of 500 inhabitants according to the last census in 2007. Historical data demonstrate that the noun Takrouna is likely to be an evolution of the word "Takrount" which is a Berber word meaning "horn". Historical data also affirm a Moroccan origin for this population (Ghalia 1994). It is important to note that Takrouna has kept until now their Berber customs in addition to the acquired Muslim Arab culture.

Sejnane is a village located in the extreme North of Tunisia, in the mountains of Khroumiries. It borders Mateur to the South, the region of South Bizerte to the east, Nefza to the west, while it faces the Mediterranean Sea to the north. Today the village is divided into seven "Imadat": Hchachna, Ababsa, Maalia, Mcharga, Shabna, Amadan and Sidi Mechreg. It covers a total of 66,000 hectares and its population, according to the last census in 2007, reaches 47,000 inhabitants. This region was occupied since the prehistory as indicated by the Iberomaurusian site of Rechada Souda in Cap Serat. The considerable number (which exceeds one hundred) of the "Houanet", a sort of "rooms" hollowed in the rocks, gives evidence demonstrating the presence of Berbers, as the autochthons of this region.

These two populations ceased to use their Berber language; they became Arabic speakers, but retained their Berber customs.

A total of 80 autochthonous Berbers, 47 from Sejnane and 33 from Takrouna, were analysed. The study was approved by the local health authorities of Bizerte and Sousse for Sejnane and Takrouna respectively. The sampling was conducted in the hospital of each village under the supervision of a sanitary staff. It was preceded by a survey for every individual. The principles of confidentiality were strictly applied during the process of sampling. An informed consent was obtained from all individuals participating in the study. In each village we sampled unrelated individuals with a level of Sejnanian or Takrounian autochthony up to three generations.

FIGURE 1. Map showing the localization of the two populations studied.

MtDNA amplification and sequencing

MtDNA was amplified using the primers L48 (5'–CTCACGGGAGCTCTCCATGC–3') and H408 (5'–CTGTTAAAAGTGCATACCGCCA–3'). The temperature profile was 95°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec, for 35 cycles of amplification. The amplified samples were purified with Microspink S-300 HR columns (Amersham Biosciences), according to the manufacturer's specifications. The sequence reactions were carried out using the kit Big-Dye Terminator Cycle Sequencing Ready Reaction (AB Applied Biosystems), with one of the above primers, in both forward and reverse directions. A protocol based on MgCl2/ethanol precipitation was used for post-sequence purification of samples, which were then run in an automatic sequencer ABI 3100. The nucleotide positions considered for the analysis were 73 to 340 for HVII.

Phylogenetic analysis

Molecular diversity indexes (H: sequence diversity is defined as the probability that two randomly chosen haplotypes are different in the sample; π: nucleotide diversity is the probability that two randomly chosen homologous nucleotide sites are different; MPD: average number of pairwise differences is the mean number of differences between all pairs of haplotypes in the sample; D_I: Tajima's statistic test is based on the infinite-site model without recombination, appropriate for short DNA sequences, it compares two estimators of the mutation parameter theta) and mismatch distributions were obtained using the software ARLEQUIN program v 2.0 package (Schneider *et al.* 2000). Sequences were aligned manually.

Phylogenetic relationships were estimated using median-joining networks version 4.5.1.6 (http: www.flux-us-engineering.com).

RESULTS

HVRII diversity in Tunisia

A total of 80 individuals from Tunisian population have shown 31 different HVII sequences (22 haplotypes in Sejnane and 9 in Takrouna) defined by 33 segregating nucleotide positions (*Table 1*). Diversity index values for the second hypervariable region show that Sejnane has a higher internal diversity than Takrouna. Compared with other populations, we note that Takrouna has the lowest sequence diversity (H=0.784).

It is widely accepted that the mismatch distribution retains valuable information about demographic episodes undergone during the history of a population (Pereira *et al.* 2000). The mismatch distribution in the two Tunisian populations for HVII shows ragged and multimodal distributions (*Figure 2*). This conclusion supports the idea that either these two Tunisian communities are ancient and stationary, or having diversified origins. Similar distributions are obtained for other African populations (Pereira *et al.* 2000, Salas *et al.* 2000). The fit to the Rogers and Harpending distribution allows the estimation of τ (this parameter consists in $\tau=\mu lt$, where μ is mutation rate per nucleotide, l is sequence length and t is time in generation after a population expansion). For Sejnane, HVIIτ_{II}=3.678, while for HVI, the value obtained was τ_I=5.67. In Takrouna, the values were τ_{II}=2.918 and τ_I=4.146 respectively. If an expansion is assumed, then the ratio τ_{II}/τ_I, together with the lengths of HVII and HVI can be used to derived μ_{II}/μ_I, which is 0.913 for Sejnane and 0.947 for Takrouna. The mean value is 0.93, a value close to that reported by Salas et al. 2000 (0.943 for Tuscan), excepting for Galicia, where the very high value found (1.845) is related to the low mean of nucleotide differences reported for HVI in that population (*Table 1*). It has been shown that mutation rate heterogeneity

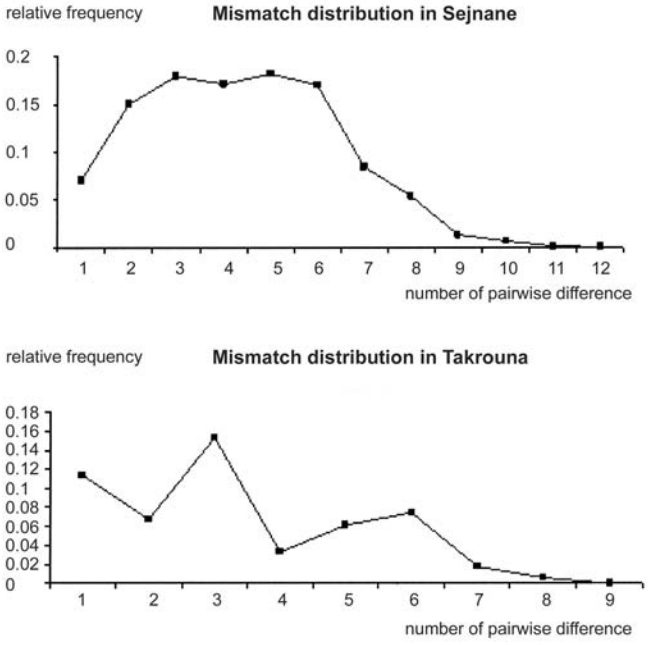


FIGURE 2. Mismatch distribution in Sejnane and Takrouna based on HVII.

TABLE 1. Results of the computation of several diversity indexes for the HVII region analyzed in several populations distributed in different continents.

Populations	**n**	**K**	**S_{II}**	**S_{II}/S_I**	**H**	π	**MPD**	**MPD_{II}/MPD_I**	**D_{II}**	**D_I**	**μ_{II}/μ_I**
Sejnane**	47	22	21	0.42	0.934	0.012	3.38	0.55	-0.936	-1.552	0.913
Takrouna**	33	9	12	0.75	0.784	0.008	2.35	1.035	-0.653	-1.411	0.947
Galician	71	47	35	0.85	0.968	0.0143	3.8	1.678	-1.53	-2.328*	1.845
British	100	51	46	0.951	0.962	0.0146	3.87	1.203	-1.783*	-2.121	0.998
French	12	10	12	-	0.97	0.0138	3.65	-	-	-	-
Tuscan	49	36	32	0.805	0.981	0.0157	4.16	1.143	-1.413	-2.06*	0.943
Bulgarian	30	24	19	0 .710	0.984	0.0127	3.36	1.021	-1.039	-1.878*	1.152
Turkish	29	27	34	0.887	0.995	0.0187	4.96	1.041	-1.568	-1.922*	1.029
Biaka Pygmy	17	14	19	1.247	0.971	0.0283	7.5	1.28	-1.318	1.219	-
Mbuti Pygmy	20	13	17	1.073	0.942	0.0188	4.99	0.807	-0.158	1.463	-
Kung	26	13	12	0.975	0.874	0.009	2.4	1.044	-0.794	-1.038	-

n: sample size; **K**: number of different sequences found; **S**: number of variable positions (S_I at the HVI region and S_{II} at the HVII one); **H**: sequence diversity; **π**: nucleotide diversity; **MPD**: average number of pairwise differences (MPDI at the HVI region and MPDII at the HVII); **D_{II}**: Tajima's statistic for HVII; **D_I**: Tajima's statistic for HVI; *: P<0.05; **: present study, the rest of data are from Salas *et al*. 2000.

may lead to an overestimate of τ (Meyer *et al*. 1999). Then, mutation rate heterogeneity does not affect the τ_{II}/τ_{I} ratio and, therefore, it seems that HVII mutates, on average, slightly faster than HVI (Salas *et al.* 2000).

Site 73

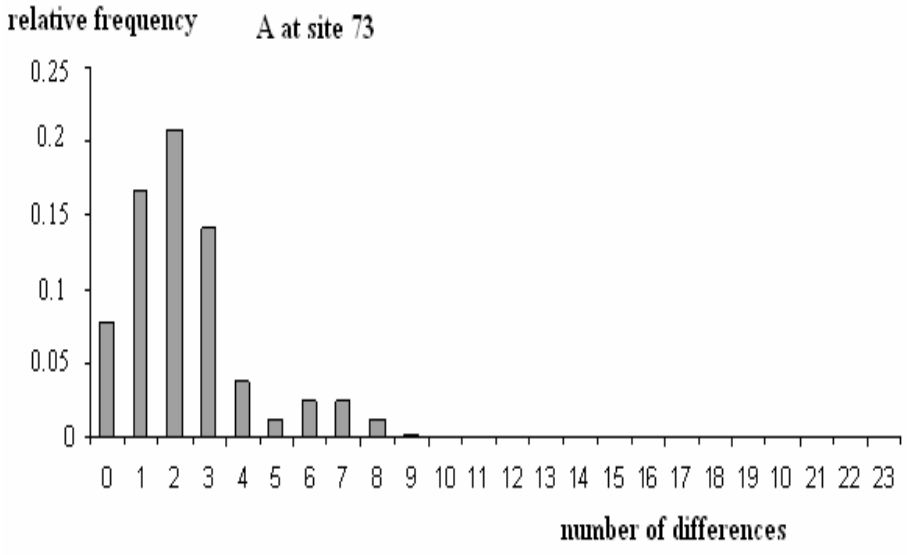
The most variable nucleotide site in our data is site 73, where the bases adenine and guanine are respectively present in 14 np 73A and 33 np 73G individuals, in the community of Sejnane and in 24 and 9 individuals in the population of Takrouna. The fact that, viewed at a world level, most populations are fixed for guanine at site 73, makes it likely that guanine is the ancestral base and adenine is the mutated one (Wilkinson *et al.* 1996).

It is striking that the mtDNA sequences with A at site 73 are considerably less diverse than those with G at that site. Counting nucleotide differences in the full data set (considering together the two populations of Sejnane and Takrouna), two sequences carrying A at site 73 have on average 2 and at most 9 observed differences (*Figure 3*). The mean and maximum numbers of pairwise observed differences among the sequences with G at site 73 are much larger: 10 and 22, respectively (*Figure 3*). This indicates that the group of individuals with A at site 73 has a much more recent common mitochondrial DNA ancestor than the group of individuals with G at that site. This systematic difference between the A and G groups regarding pairwise sequence diversity also constitutes further evidence that the high variation at site 73 confirms that G is probably the ancestral nucleotide (Wilkinson *et al.* 1996).

We analyzed the phylogenetic structure of the Tunisian mitochondrial gene pool using a reduced median network relating in the first case to HVI sequences from the two populations studied. In the second case we incorporate for every population HVII site 73 in the network (*Figures 4* and *5*).

In the community of Takrouna we remark that when we incorporate site 73, the structure of the network is greatly clarified and the different haplogroups are seen much more distinctly.

In the community of Sejnane, the G to A substitution at site 73 splits group 1 from group 2. The group 1 sequences being characterized by an A at site 73 while group 2 carries



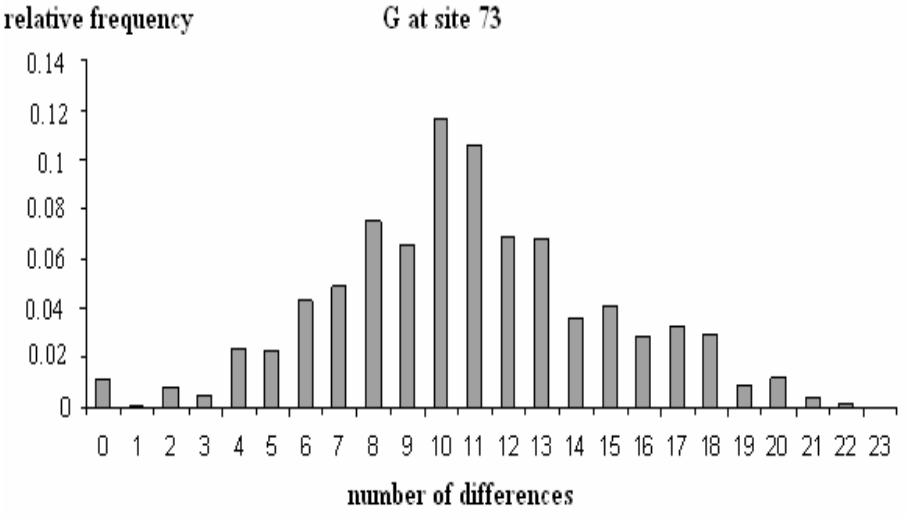


FIGURE 3. Histograms of the numbers of pairwise mtDNA sequence differences amongst the individuals carrying A and G at site 73.

a G at site 73. We note that group 1 contains haplogroups H and V. The central type in group 1 (indicated by an asterisk) is the reference sequence by Anderson *et al.* (1981). It is striking that the phylogeny of group 1 is clearly star-like, while that of group 2 is not (*Figure 5*). This provides strong evidence of a relatively recent, major population expansion which appears to have been confined to group 1 only.

Using a mutation rate for hypervariable region I of 1 in 21,000 years as in Richards *et al.* (1996), the minimum ages of sequences with A at site 73 in Sejnane (group 1) and in Takrouna are 28,000 years and 21,000 years respectively. These values are closer to these found by Wilkinson-Herbots *et al.* (1996), when studying European population. The value is 24,500 years, reflecting the population expansion following the last glacial maximum about 20,000 BP.

Microsatellite 303-315

In the present study we have investigated the polymorphism of the mitochondrial microsatellite, situated between 303 and 315 nps in two Tunisian populations. Polymorphism at both populations was compared with published data: Portuguese (Pereira *et al.* 2000); Italians (Tagliabracci *et al.* 2001); French (Rousselet *et al.* 1998); British (Piercy *et al.* 1996); Spanish (Crespillo *et al.* 2000); Turkish (Calafell *et al.* 1996); Moroccan (Rando *et al.* 1998); Angolares, Forros and Tongas (Trovoada *et al.* 2003); Khwe and Kung (Chen *et al.* 2000). The obtained results (*Figure 6*) reveal that:

– Sejnane presents 4 haplotypes: haplotype 309-315.1 (51%); haplotype 309.1-315.1 (36%); haplotype associated with the Cambridge Reference Sequence (CRS) 309-315 (4%) and haplotype 309.2-315.1 (9%).

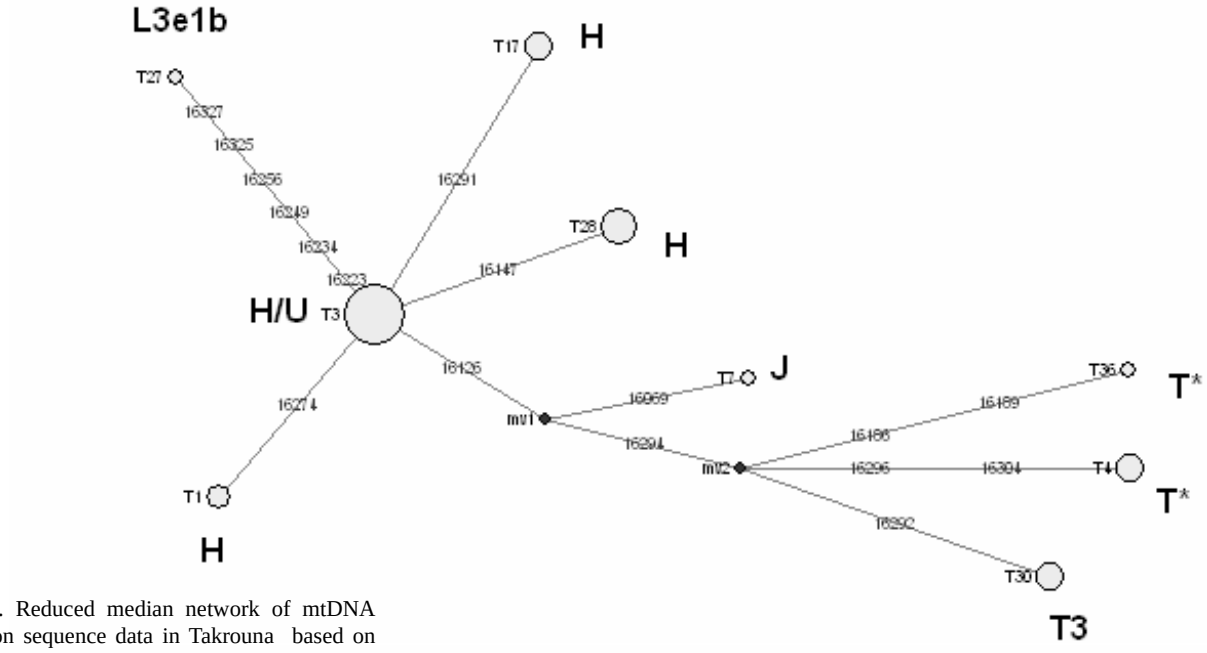


FIGURE 4a. Reduced median network of mtDNA control region sequence data in Takrouna based on hypervariable segment I.

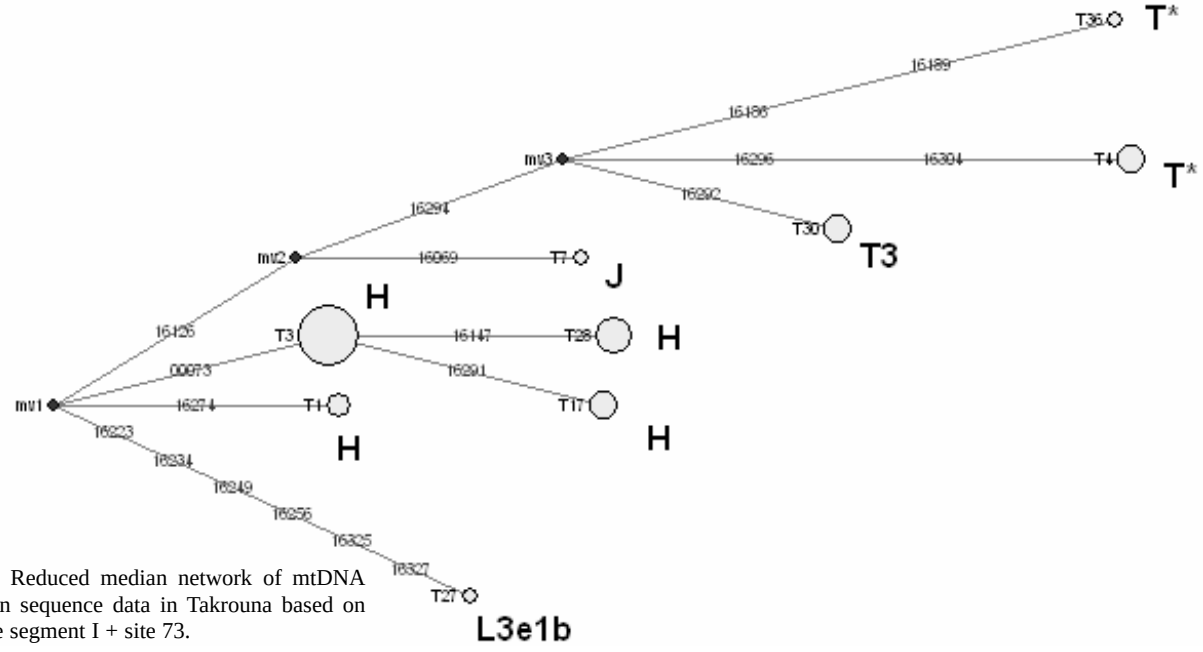


FIGURE 4b. Reduced median network of mtDNA control region sequence data in Takrouna based on hypervariable segment I + site 73.

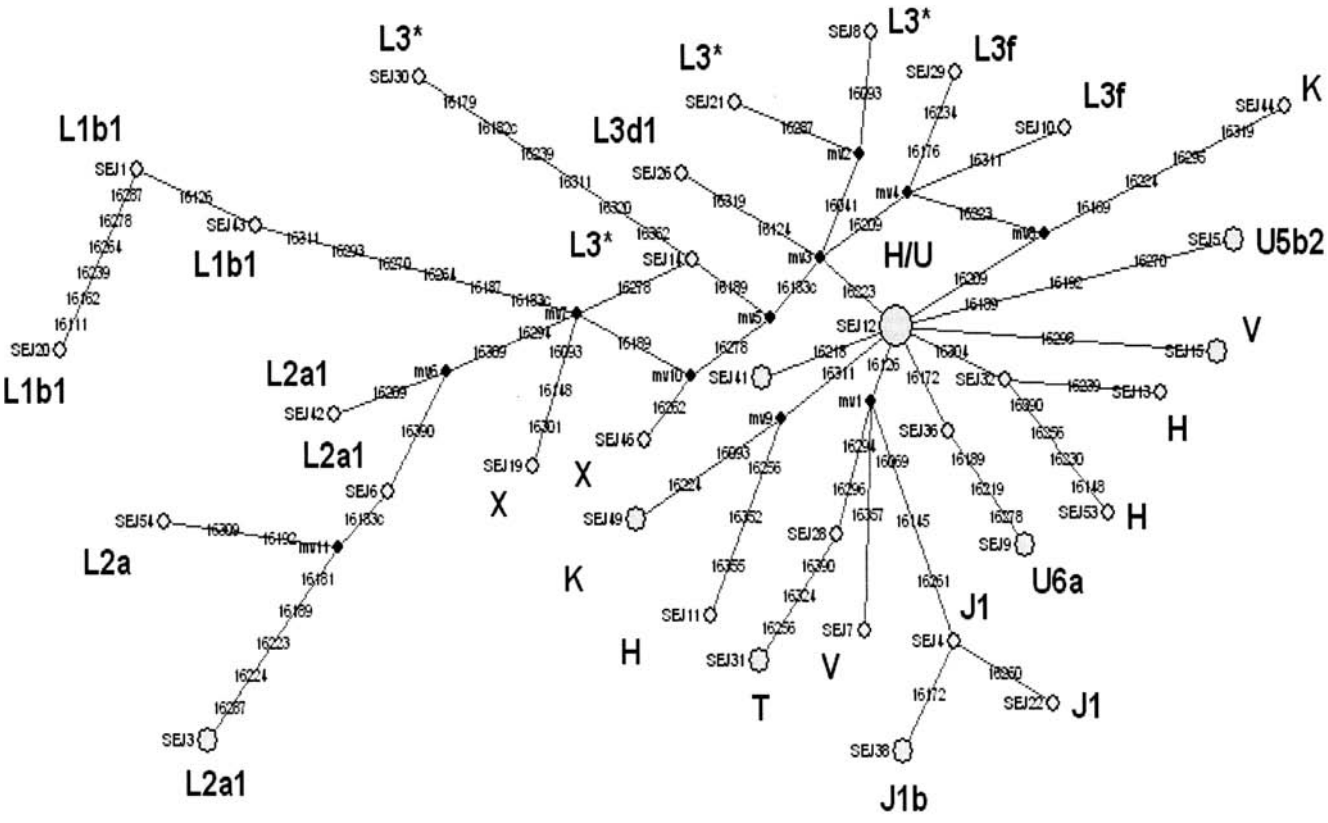


FIGURE 5a. Reduced median network of mtDNA control region sequence data in Sejnane based on hypervariable segment I.

– The community of Takrouna presents three haplotypes: haplotype 309-315.1 (46%); haplotype 309.1-315.1 (27%) and the haplotype 309.2-315.1 (27%).
– The two haplotypes 309.1-315.1 and 309-315.1 are the most frequent in all studied populations.
– The haplotype associated with the Cambridge Reference Sequence (CRS) is only detected in the Berbers from Sejnane, in the sub-Saharan populations of Khwe, Kung and Angolares, and is also detected in the British people.

In fact, we note that the two frequent haplotypes are associated with the majority of haplogroups studied (*Table 2*), despite their phylogenetic divergence. This result may be explained by the absence of linkage disequilibrium between the site of the microsatellite and the rest of the mtDNA molecule. It is also explained by the mutation rate and reversion of microsatellites. Nevertheless, knowing the fast rate of evolution of microsatellite markers, we look forward to a greater number of haplotypes than found. The relatively reduced number found in sub-Saharan populations, which exhibit phylogenetically old haplogroups, can be explained by the factor of selection in a functional context. The diversity of haplotypes in this microsatellite in Eurasian populations is associated with haplogroups H and V which are more recent.

DISCUSSION

In this work we assessed human genetic diversity by focusing on two Berber communities from North Tunisia with very different population size: the small village of Takrouna (500 inhabitants), and the village of Sejnane (47,000 inhabitants). We sampled 33 unrelated healthy individuals in Takrouna representing each family of the village. Increasing the number of individuals would have implied the selection of related individuals which would have erroneously increased the frequency of some haplotypes. For the population of Sejnane, we analyzed 47 unrelated individuals representing 1/1000 of the total number of inhabitants. The aim of this work was just to investigate polymorphisms of HVII in these two Tunisian Berber communities and to compare our results with others.

Our work is the first, at our knowledge, studying the polymorphisms in HVII and illustrating the phylogenetic importance of site 73 in North African populations. Our results show that site 73 is very informative when studying two North-African populations. The same results are obtained when studying European populations (Richards *et al.* 1996, Wilkinson *et al.* 1996). The topology of the network relating to a variety of European data on hypervariable region I is greatly refined when incorporating site 73. Wilkinson *et al.* (1996) suggested that the mtDNA lineages with A at site 73 could have their origin in a small glacial refuge population which expanded after the last

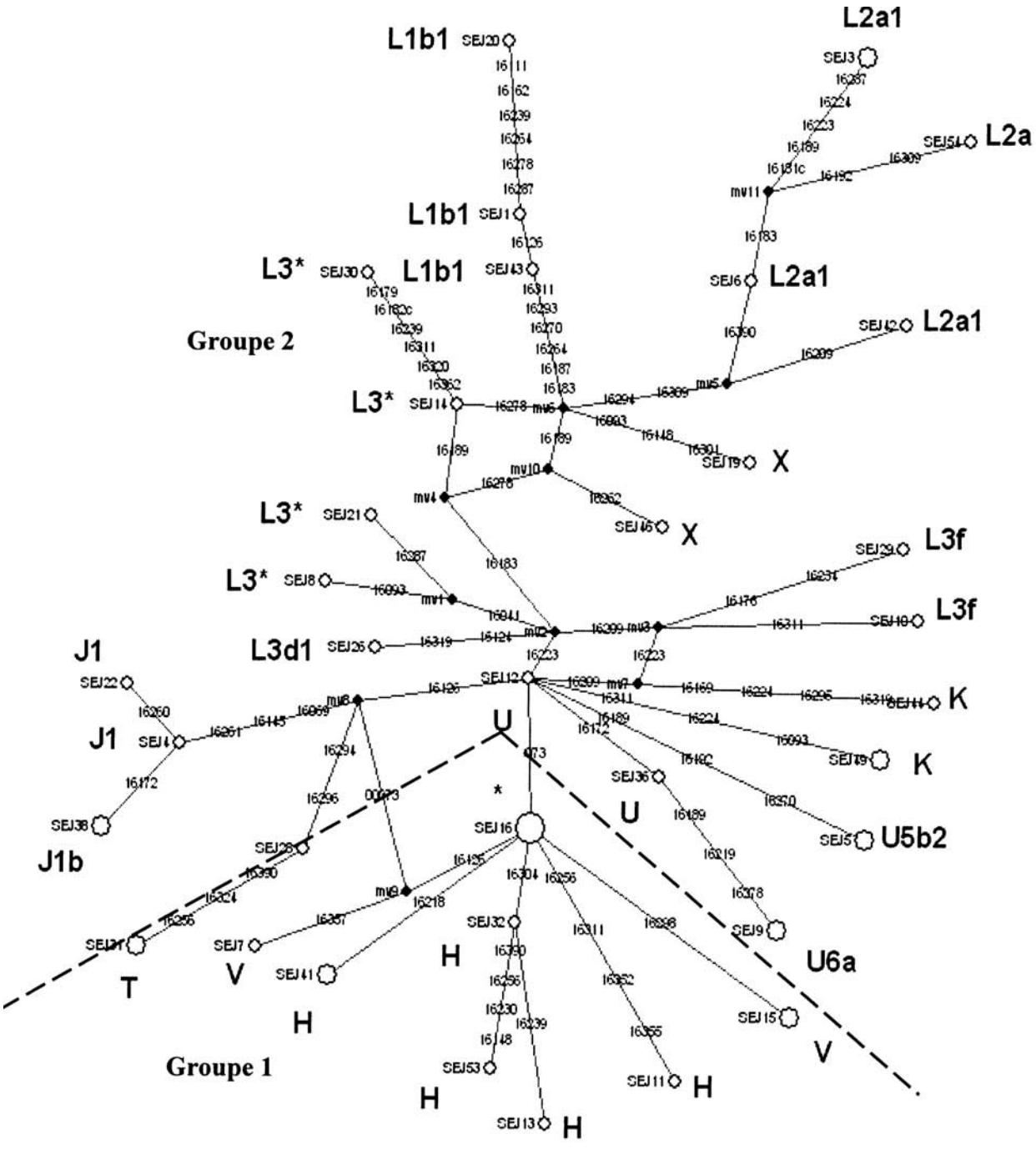


FIGURE 5b. Reduced median network of mtDNA control region sequence data in Sejnane based on hypervariable segment I + site 73.

glacial maximum around 20,000 BP. Compared to other studies (Richards *et al.* 1996, Wilkinson *et al.* 1996) we conclude that site 73 contains valuable information on the history of both North African and European populations.

Within the HVII, a region of microsatellite-like sequence can be found (position 303-315). These short tandem repeats, particularly a C-mononucleotide track interrupted by a single thymidine at position 310, have been shown to exhibit length polymorphism among individuals, as well as variation within an individual, which accompanies the process of aging and cancer (Michikawa *et al.* 1999, Liu *et al.* 2003). This microsatellite 303-315 is also involved in the formation of a persistent RNA-DNA hybrid that leads to the initiation of mtDNA heavy-strand replication.

As the polycytosine (poly-C) tracts of hypervariable region II require further analyses, we focused our research mainly on the study of polymorphism of the microsatellite 303-315. In our study, in haplogroup H we note the presence of 4 haplotype microsatellites (*Table 2*), whereby 309-315 is the shortest and 309.2-315.1 the longest. These two extreme haplotypes are present in haplogroups H and V. These data can be explained in two ways:

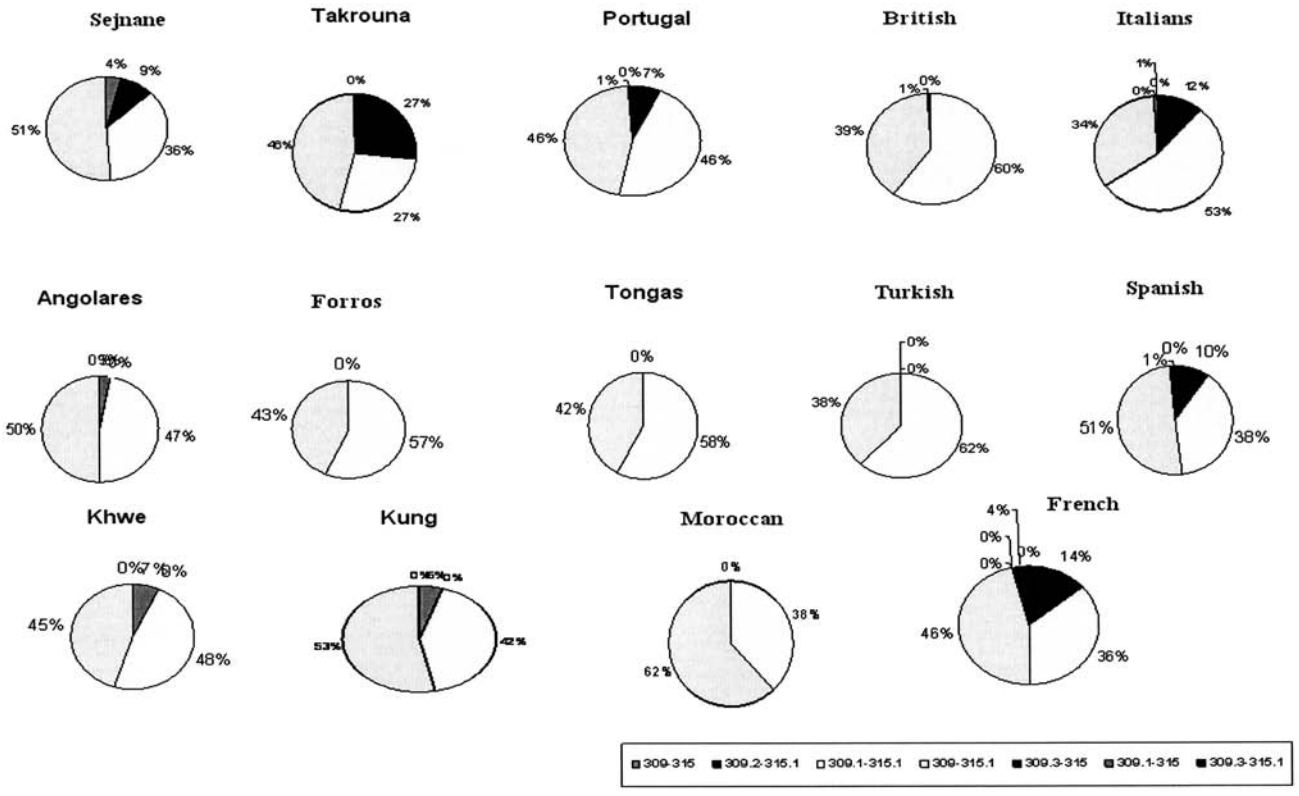


FIGURE 6. Distribution of 303-315 mitochondrial haplotypes in studied populations.

– Either the natural selection conducted against the extreme forms did not operate, on one hand because of the recent character of the two haplogroups and on the other hand due to the slowness of the processes.
– Or, the structure of the recent haplogroups H and V gives a sufficient stability to the mtDNA molecule permitting a great fluctuation of size in the microsatellite marker considered.

To distinguish between these two hypotheses, a deep analysis considering the fine characterisation of mtDNA H haplogroup will be done in these two Tunisian communities.

Our results show this mitochondrial microsatellite as a frequent hot spot of mononucleotide insertions. Our results also confirm the high diversity of the population of Sejnane because four haplotypes for the studied microsatellite are detected in this population at frequencies between 4% and 51% whereas the majority of the other populations exhibit 2 or 3 haplotypes.

We must note that Mambo *et al*. (2003) demonstrated recently that the D310 region is highly susceptible to mutations induced by exposure to the oxidant agent tert-butyl hydroperoxide. These findings may explain the high frequency of homoplasmic D310 somatic mutations in many tumor types.

In conclusion, the results of the present study demonstrate the valuable benefits of typing HVII and its use in human population study.

ACKNOWLEDGEMENTS

We gratefully acknowledge blood donors whose availability made this study possible. This research was supported by the Tunisian Ministry of Research, Technology and Development of Competences.

TABLE 2. Association between haplogroups and the different haplotype microsatellites found in the two populations of Sejnane and Takrouna. In bold are the shortest (309-315) and the longest (309.2-315.1) haplotypes.

Haplogroups	Takrouna	Sejnane
H	309-315.1 **309.2-315.1** 309.1-315.1	**309-315** 309-315.1 309.1-315.1
T	309.1-315.1 309-315.1	309-315.1
J	309-315.1	309.1-315.1 309-315.1
L3e1b	309.1-315.1	
L1b1		309.1-315.1 309-315.1
L2a		309-315.1
L2a1		309.1-315.1 309-315.1
L3*		309-315.1 309.1-315.1
L3f		309-315.1
L3d1		309-315.1
V		**309-315** **309.2-315.1**
X		309-315.1
K		309.1-315.1
U		309-315.1
U5b2		309.1-315.1
U6a		**309.2-315.1**

*: reference sequence by Anderson *et al*. (1981)

REFERENCES

ANDERSON S., BANKIER A. T., BARRELL B. G., DE BRUIJN M. H., COULSON A. R., DROUIN J., EPERON I. C., NIERLICH D. P., ROE B. A., SANGER F., SCHREIER P. H., SMITH A. J., STADEN R., YOUNG I. G., 1981: Sequence and organization of the human mitochondrial genome. *Nature* 290: 457–465.

BANDELT H. J., FORSTER P., SYKES B. C., RICHARDS M. B., 1995: Mitochondrial portraits of human populations using median networks. *Genetics* 141: 743–753.

BROWN W. M., GEORGE M. Jr., WILSON A. C., 1979: Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. U.S.A.* 76: 1967–1971.

CALAFELL F., UNDERHILL P., TOLUN A., ANGELICHENA D., KALAY-DJIEVA L., 1996: From Asia to Europe: mitochondrial DNA sequence variability in Bulgarians and Turks. *Annals of Human Genetics* 60: 35–49.

CERNY V., CONNIE J. M., JAKUB R., MARTINA Z., CHRISTOPHER M. E., MARTIN H., LUISA P., 2008: Regional differences in the distribution of the sub-Saharan, West Eurasian, and South Asian mtDNA lineages in Yemen. *Am. J. Phys. Anthropol*. 136, 2: 128–137.

CHEN Y. S., OLCKERS A., SCHURR T. G., KOGELNIK A. M., HUOPONEN K., WALLACE D. C., 2000: MtDNA variation in the South African Kung and Khwe and their genetic relationship to other African populations. *Am. J. Hum. Genet.* 66: 1362–1383.

COUDRAY C., OLIVIERI A., ACHILLI A., PALA M., MELHAOUI M., CHERKAOUI M., EL-CHANNOUFI F., KOSSMANN M., TORRONI A., DUGOUJON M., 2009: The complex and diversified mitochondrial gene pool of Berber populations. *Annals of Human Genetics* 73, 2: 196–214.

CRESPILLO M., LUQUE J. A., PAREDES M., FERNANDEZ R., RAMIREZ E., VALVERDE J. L., 2000: Mitochondrial DNA sequences for 118 individuals from northeastern Spain. *International J. of Legal Medicine* 114, 1-2: 130–132.

CROTEAU D. L., BOHR V. A., 1997: Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. *J. of Biological Chemistry* 272: 25409–25412.

HAUSWIRTH W. W., CLAYTON D. A., 1985: Length heterogeneity of a conserved displacement loop sequence in human mitochondrial DNA. *Nucleic Acids Research* 13: 8093–8104.

LEE H. Y., CHUNG U., YOO J. E., PARK M. J., SHIN K. J., 2004: Quantitative and qualitative profiling of mitochondrial DNA length heteroplasmy. *Electrophoresis* 25: 28–34.

LEE D. Y., CLAYTON D. A., 1998: Initiation of mitochondrial DNA replication by transcription and R-loop processing. *J. of Biological Chemistry* 273: 30614–30621.

LIU V. W., YANG H. J., WANG Y., TSANG P. C., CHEUNG A. N., CHIU P. M., NG T. Y., WONG L. C., NAGLEY P., NGAN H. Y., 2003: High frequency of mitochondrial genome instability in human endometrial carcinomas. *British J. of Cancer* 89: 697–701.

MAMBO E., GAO X., COHEN Y., GUO Z., TALALAY P., SIDRANSKY D., 2003: Electrophile and oxidant damage of mitochondrial DNA leading to rapid evolution of homoplasmic mutations. *Proc. Natl. Acad. Sci. U.S.A.* 100: 1838–1843.

MEYER S., WEISS G., HAESELER A. V., 1999: Pattern of nucleotide substitution and rate heterogeneity in the hypervariable regions I et II of human mtDNA. *Genetics* 152: 1103–1110.

MICHIKAWA Y., MAZZUCCHELLI F., BRESOLIN N., SCARLATO G., ATTARDI G., 1999: Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. *Science* 286: 774–779.

PEREIRA L., PRATA M. J., AMORIM A., 2000: Diversity of mtDNA lineage in Portugal: not a genetic edge of European variation. *Annals of Human Genetics* 64: 491–506.

PIERCY R., SULLIVAN K. M., BENSON N. B., GILL P., 1996: The application of mitochondrial DNA typing to the study of white Caucasian genetic identification. *International J. of Legal Medicine* 106: 85–90.

RANDO J. C., PINTO F., GONZALEZ A. M., HERNANDEZ M., LARRUGA J. M., CABRERA V. M. BANDELT H. J., 1998: Mitochondrial DNA analysis of northwest African populations reveals genetic exchanges with European, near-eastern, and sub-Saharan populations. *Annals of Human Genetics* 62: 531–550.

RICHARDS M. B., MACAULAY V., HICKEY E., VEGA E., SYKES B., GUIDA V., RENGO C. et al., 2000: Tracing European founder lineages in the Near Eastern mitochondrial gene pool. *Am. J. Hum. Genet.* 67: 1251–1276.

RICHARDS M. B., CORTE REAL H., FORSTER P., MACAULAY V., WILKINSON-HERBOTS H. M., DEMAINE A.,

PAPIHA S., HEDGES R., BANDELT H. J., SYKES B., 1996: Paleolithic and Neolithic lineage in the European mitochondrial gene pool. *Am. J. Hum. Genet*. 59: 185–203.

ROUSSELET F., MANGIN P., 1998: Mitochondrial DNA polymorphisms: a study of 50 French Caucasian individuals and application to forensic casework. *International J. of Legal Medicine* 111, 6: 292–308.

SALAS A., LAREU V., CALAFELL F., BERTRANPETIT J., CARRACEDO A., 2000: MtDNA hypervariable region II (HVII) sequences in human evolution studies. *European J. of Human Genetics* 8: 964–974.

SANCHEZ-CESPEDES M., PARRELLA P., NOMOTO S., COHEN D., XIAO Y., ESTELLER M., JERONIMO C., JORDAN R. C., NICOL T., KOCH W. M., SCHOENBERG M., MAZZARELLI P., FAZIO V. M., SIDRANSKY D., 2001: Identification of a mononucleotide repeat as a major target for mitochondrial DNA alterations in human tumors. *Cancer Research* 61: 7015–7019.

TAGLIABRACCI A., TURCHI C., BUSCEMI L., SASSAROLI C., 2001: Polymorphism of the mitochondrial DNA control region in Italians. *International J. of Legal Medicine* 114, 4-5: 224–228.

TROVOADA M. J., PEREIRA L., GUSMAO L., ABADE A., AMORIM A., PRATA M. J., 2003: Pattern of mtDNA variation in three populations from Sao Tomé e Principe. *Annals of Human Genetics* 68: 40–54.

WATSON E., FORSTER P., RICHARDS M., BANDELT H. J., 1997: Mitochondrial footprints of human expansions in Africa. *Am. J. Hum. Genet*. 61: 691–704.

WILKINSON-HERBOTS H. M., RICHARDS M. B., FORSTER P., SYKES B. C., 1996: Site 73 in hypervariable region II of the human mitochondrial genome and the origin of European populations. *Annals of Human Genetics* 60: 499–508.

WILSON M.R., STONEKING M., HOLLAND M. M., DI ZINNO J. A., BUDOWLE B., 1993: Guidelines for the use of mitochondrial DNA sequencing in forensic science. *Crime Laboratory Digest* 20: 68–77.

Sabeh Frigi
Hajer Ennafaa
Lotfi Cherni
Amel El Gaaied
Laboratory of Molecular genetics, Immunology and Human Pathology
Faculty of Sciences of Tunis
University El Manar
1060 Tunis, Tunisia
E-mail: sabehfrigi@yahoo.fr
ennafaahajer@yahoo.fr
c_l22@yahoo.fr
a_benammarelgaaied@yahoo.fr

APPENDIX 1. Population of Sejnane.

No. of haplotype	N=47(100%)	Characteristic mutations in HVI	Characteristic mutations in HVII	Haplogroups
1	1 (2.2)	126 187 189 223 264 270 278 293 311	073 152 182 185G/T 189 195 247 263 309.1 315.1 357	L1b1
2	1(2.2)	111 126 162 187 189 223 239 270 278 293 311	073 146 152 182 185G/T 189 247 263 315.1 357	L1b1
3	1(2.2)	187 189 223 264 270 278 293 311	073 152 182 185G/T 195 247 263 315.1 357	L1b1
4	1(2.2)	189 192 223 278 294 390	073 143 146 152 195 263 315.1	L2a
5	2 (4.2)	181 223 278 287 294 309 390	073 143 146 152 195 263 309.1 315.1	L2a1
6	1(2.2)	183**A/C** 189 223 278 294 309 390	073 146 152 195 198 263 315.1	L2a1
7	1(2.2)	183**A/C** 189 209 223 278 294 309	073 143 146 152 195 263 309.1 315.1	L2a1
8	1(2.2)	041 093 223	073 150 263 315.1	L3*
9	1(2.2)	183**A/C** 189 223	073 146 153 263 309.1 315.1	L3*
10	1(2.2)	041 223 278	073 150 263 315.1	L3*
11	1(2.2)	179 182**A/C** 183A/C 189 223 239 311 320 362	073 150 199 204 263 309.1 315.1	L3*
12	1(2.2)	209 223 311	073 189 200 263 315.1	L3f
13	1(2.2)	176 209 223 234	073 189 263 315.1 318	L3f
14	1(2.2)	124 223 319	073 146 152 195 263 315.1	L3d1
15	1(2.2)	256 311 352 355	146 263 310del	H
16	1(2.2)	239 304	263 315.1	H
17	1(2.2)	304	263 315.1	H
18	1(2.2)	148 230 256 304 390	150 207 263 309.1 315.1	H
19	2 (4.2)	218	263 309.1 315.1	H
20	1(2.2)	CRS	195 263 309.1 315.1	H
21	2 (4.2)	CRS	263 309.1 315.1	H
22	2(4.2)	CRS	263 315.1	H
23	1(2.2)	069 126 145 261	073 152 295 309.1 315.1	J1
24	1(2.2)	069 126 145 260 261	073 152 263 295 315.1	J1
25	2(4.2)	069 126 145 172 261	073 242 263 295 315.1	J1b
26	1(2.2)	126 357	072 263 310del	V
27	2 (4.2)	298	072 195 263 309.2 315.1	V
28	1(2.2)	093 148 183A/C 189 223 278 301	073 153 195 225 226 263 315.1	X
29	1(2.2)	183**A/C** 189 223 262 278	073 153 195 225 263 315.1	X
30	1(2.2)	169 209 224 295 319	073 152 263 309.1 315.1	K
31	2 (4.2)	093 224 311	073 150 199 204 263 309.1 315.1	K
32	1(2.2)	126 294 296	073 263 315.1	T
33	2 (4.2)	126 256 294 296 324 390	073 263 315.1	T
34	1(2.2)	CRS	073 263 315.1	U
35	1(2.2)	172	073 263 315.1	U
36	2 (4.2)	189 192 270	073 150 263 309.1 315.1	U5b2
37	2 (4.2)	172 189 219 278	073 189 263 309.2 315.1	U6a

*: reference sequence by Anderson *et al*. (1981)

APPENDIX 2. Population of Takrouna.

No. of haplotype	**N=33(100%)**	**Characteristic mutations in HVI**	**Characteristic mutations in HVII**	**Haplogroups**
1	2 (6)	274	263 315.1	H
2	3 (9.1)	CRS	263 309.2 315.1	H
3	5 (15.1)	147	263 315.1	H
4	3 (9.1)	CRS	263 315.1	H
5	3(9.1)	291	263 309.1 315.1	H
6	6 (18.1)	CRS	152 195 263 309.2 315.1	H
7	2 (6)	CRS	195 263 309.1 315.1	H
8	3 (9.1)	126 294 296 304	073 151 263 309.1 315.1	T
9	3 (9.1)	126 292 294	073 146 152 183 263 279 315.1 374	T
10	1(3.1)	126 186 189 294	073 152 263 315.1	T
11	1(3.1)	069 126	073 150 195 263 295 315.1	J
12	1(3.1)	223 234 249 256 325delG 327	073 150 189 200 263 309.1 315.1	L3e1b