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Paleo-mtDNA analysis and population genetic aspects of old Thracian populations from South-East of Romania

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ABSTRACT: Paleo-mtDNA analysis and population genetic aspects of old Thracian populations from South-East of Romania. We have performed a study of mtDNA polymorphisms (HVR I and HVR II sequences) on the skeletal remains of some old Thracian populations from SE of Romania, dating from the Bronze and Iron Age in order to show their contribution to the foundation of the modern Romanian genetic pool and the degree of their genetic kinships with other old and modern human European populations. For this purpose we have applied and adapted three DNA extraction methods: the phenol/chloroform, the guanidine isotiocianat and silica particles and thirdly the Invisorb Forensic Kit (Invitek)-based DNA extraction method. We amplified by PCR short fragments of HVR I and HVR II and sequenced them by the Sanger method.

So far, we have obtained mtDNA from 13 Thracian individuals, which we have compared with several modern mtDNA sequences from 5 European present-day populations.

Our results reflect an evident genetic similarity between the old Thracian individuals and the modern populations from SE of Europe.

KEY WORDS: mtDNA HVR I, aDNA, Thracian population

The scientific research in the field of ancient and degraded DNA has opened new possibilities and prospects for generally studying the evolution of life and studying the evolution of humankind in particular and also in forensic sciences. The major sources of biological material to examine the past are skeletons and rarely some soft tissues (especially in case of mummies).

The mitochondrial DNA (mtDNA) markers are often used in such studies because of some particular traits of mt genome, such as: its presence in multiple copies per cell; its maternal inheritance; lack of recombination; and its mutation rate about 10 times faster than the average nuclear genes. From the whole mt genome, the most used in population or phylogenetic studies is the D-loop region or the control region, an uncoding sequence of about 900 bp in the human mt genome. This mtDNA region shows the highest sequence

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polymorphism from the whole mt genome and mainly consists of HVR I and HVR II, which are the highly valuable DNA markers for such kind of studies.

In the human mt genome, the HVR I consists of 341 bp between the nucleotide position (np) 16,024 bp to np 16,365 and the HVR II consists of 267 bp from np 73 to np 340. The two HVR mt regions reveal about 3 % variability between two unrelated individuals.

In this context our study has focused on the mtDNA analysis (HVR I and HVR II region polymorphisms) on the skeletal remains of individuals from some old Thracian populations found in SE of Romania, dating from the Bronze and Iron Age, in order to show their genetic kinship with other old and modern European populations and their contribution to the foundation of the modern Romanian genetic pool.

Historical context

From archaeological and anthropological sources it is known that the Thracian people were formed during a long historical lapse of time by an admixture of aboriginal and new arrived human groups. By a progressive development, these people had constituted in their basic elements during the 3rd - 2nd millennium B.C.

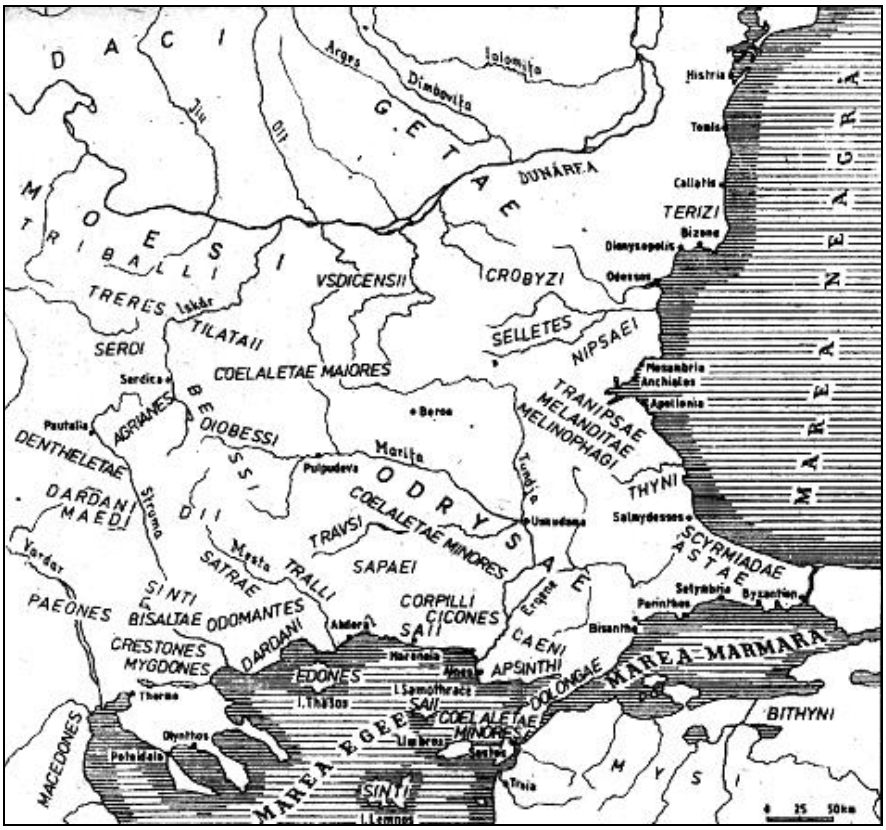

Fig. 1. The Thracian tribes (by Opermann, 1988).

The Thracian people lived on the land between the Carpathian Mountains (towards the N) and the Aegean Sea (towards the S), yet some Thracian elements were also found northwest of Little Asia and Slovakia (Fig. 1).

There is also a lot of archaeological material ascribed to the Thracian populations discovered in SE and E of Romania [1].

Material and methods

The biological material from our study has been represented by bones and teeth belonging to some individuals from the old Thracian populations from SE of Romania, which have either been well or less preserved, depending on the environmental factors from the archaeological site.

The human fossil bones of 20 individuals dating about 3200-4100 years, from the Bronze Age, belonging to some cultures such as Tei, Monteoru and Noua, were found in

graves from some necropoles in SE of Romania, namely in Zimnicea, Smeeni, Candesti, Cioinagi-Balintesti, Gradistea-Coslogeni and Sultana-Malu Rosu.

The human fossil bones and teeth of 27 individuals from the early Iron Age, dating from the 10^{th} -7^{th} century B.C. from the Hallstatt Era (the Babadag Culture), were found extremely SE of Romania near the Black Sea coast, in some settlements from Dobrogea, namely: Jurilovca, Satu Nou, Babadag, Niculitel and Enisala-Palanca.

They had fossilized in some common pits, most of them on stone surfaces, covered by loess and an ash layer. Most of the fossil teeth were much better preserved than many human old bones probably due to the enamel layer which protect teeth against damaging induced by diagenetic factors.

All human fossils from our study have been preserved at room temperature since their discovery between the years 1997-2003.

Sample preparation and DNA extraction

First and foremost, to prevent any contamination, sterile protective equipment, instruments, reagents and three different rooms for ancient DNA (aDNA) extraction, PCR amplification and cleaning-up the PCR products were used.

To remove any previous contamination, the skeletal samples were treated by firstly removing the outer layer, secondly exposing them to UV light for 10 minutes on each side, thirdly washing them with absolute EtOH, EtOH 70 %, distilled water, and lastly letting them dry at 30 °C in the oven over night. Once again, the skeletal samples were exposed to UV light for 10 minutes on each side and then ground to a fine powder.

For aDNA extraction we used the three following methods:

- the phenol-based DNA extraction method as described by Hummel [2] and modified it by removing the decalcification step and prolonging the cell lysis step to15-18 hours;
- the guanidine tiocianat and silica-based method described by Hoss and Pääbo [3] and modified it by prolonging the extraction step to 15-18 hours and removing the final elution step. The DNA extracts with silica suspension were used for the DNA amplification by PCR reactions;
- the Invisorb Forensic Kit (Invitek)-based DNA extraction method modified it by using silica particles instead of carrier suspension.

To detect any contamination of the reagents, a negative control (without sample) was used for each aDNA extraction.

The mtDNA amplification

Mitochondrial HVR I and HVR II DNA regions were amplified by single and duplex PCR reactions, each of them being amplified in two fragments, with different sets of primers, as follows:

- for the HVR I region
- the 195 bp fragment:
  1: 5'- TGACTCACCCATCAACAACC-3'
  2: 5'- GTGGCTTTGGAGTTGCAGTT-3'
- the 188 bp fragment:
  3: 5'- AACTGCAACTCCAAAGCCAC-3'
  4: 5'- TCCGTGAAATCAATATCCCG-3'
- for the HVR II
- the 100 bp fragment:

1.2: 5'- TTGGTATTTTCGTCTGGGG -3'
2.2: 5'- AGGATGAGGCAGGAATCAAA-

- the 95 bp fragment:

3.2: 5'- CCCTATGTCGCAGTATCTGTCTTT-3'
4.2: 5'- CACACTTTAGTAAGTATGTTCGCCT-3'.

The reactant mixture consisted of: 15 µl DNA extract, 5 µl PCR buffer (10x), 1.5 µl $MgCl_2$ (50 mM), 1 µl dNTP (10 mM), 0.5 µl of each primer (10 µM), 0.3 µl Tag-polymerase (5 U/mL), 3 µl BSA (Bovine Serum Albumin) (10 mg/ml), 7.5 to 15 µl of DNA extracts and sterile distilled water providing a final volume of 50 µl.

The PCR reaction profile consisted of an initial step of 3 min at 94 °C, followed by 35 – 40 cycles (45 cycles for negative controls) of: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; the final step was performed at 72 °C for 7 min.

The test for inhibitors consisted of amplifying the control DNA (DNA K_{562}) in the presence of variable amounts of aDNA extracts.

In order to prove the human origin of the amplified aDNA, a sequence of 120 bp from the control region V has been amplified, as described by Cattaneo [4].

All the PCR reactions were carried out in a Perkin Elmer GeneAmp PCR System 9600 Thermal Cycler. In order to monitor for contamination events, at least one blank control without aDNA extract was used for each PCR.

The presence of PCR products was demonstrated by UV visualisation by electrophoresis on agarose gels 2 % and photographed with a digital camera.

The DNA sequencing, alignment and statistical analysis

The mtDNA PCR products were sequenced at MWG by the Sanger method [5], after cleaning them with the MBS spin PCRapace Kit (50) (Invitek). Every aDNA sequence was amplified and sequenced at least twice and was compared with the DNA handle's sequences and the control DNA in order to detect any contamination.

The mtDNA polymorphism analysis has been made by comparing the aDNA sequences with the modern European reference sequence, Cambridge Reference Sequence (CRS) [6] and several modern mtDNA sequences from 5 European present-day populations, that is Romanian (our unpublished data), Greek, Bulgarian, Albanian and Italian populations [7] (20 DNA sequences out of each population), using the Bioedit Programme (Version 5.0.9.; Tom Hall, Departament of Microbiology, North Carolina State University) [8].

As for the statistical analysis, the small number of Thracian mtDNA sequences does not yet allow us to do a complex biostatistical analysis, so we made a rush and simple one to present only a bias of genetic kinship of old Thracian population from Romania with other modern European populations.

Thus, we calculated the percentage of point mutations in common nucleotide positions as S x 100 / N x n (S = number of point mutations; N = number of individuals from one population; n = number of nucleotide positions) and the percentage of common point mutations of the present-day European populations with the Thracian population as Im x 100 / N x n (Im = number of individuals from modern populations who show common mutations with Thracians; N and n as above).

Results and discussion

The four DNA sequences have been amplified (Fig. 2, 3) from extracts obtained by all three DNA extraction methods described above.

Up to now we have obtained aDNA from 6 individuals from the Bronze Age and from 7 individuals from the Iron Age. The other samples still require further extractions and/or amplifications in order to confirm the results.

Only the HVR I polymorphisms are presented below because we have so far only obtained a few and short Thracian HVR II sequences, without scientific relevance for a sequence analysis.

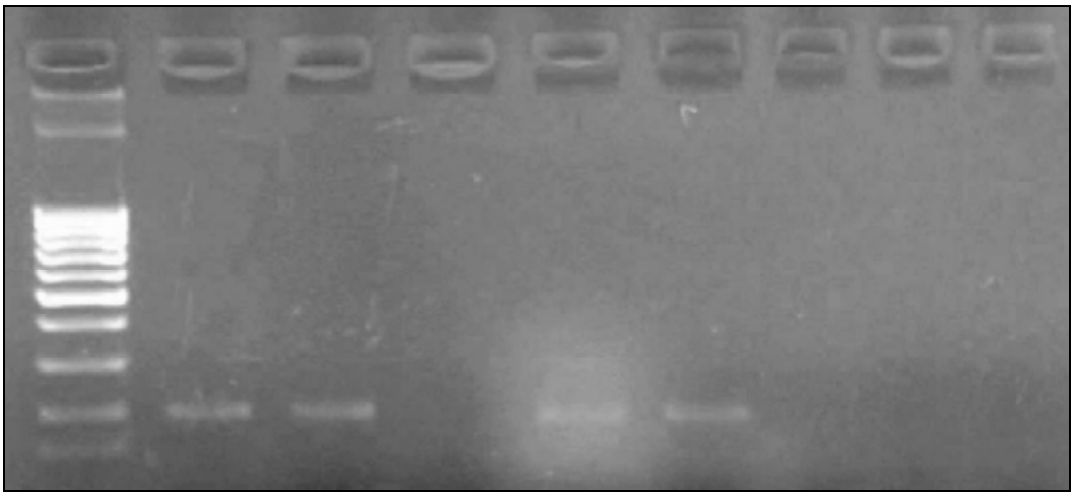

Fig. 2 The PCR amplification of the mtDNA HVR I region – the 195 bp fragment - from different aDNA extracts obtained by the guanidine tiocianat and silica-based method: Line 1 :weight molecular marker 100 bp DNA; Lines 2,3,4: different DNA extracts from the 7BA sample; Lines 5,6: different DNA extracts from the 5BA sample; Lines 7,8: negative controls of different DNA extractions; Line 9: negative control of PCR reaction

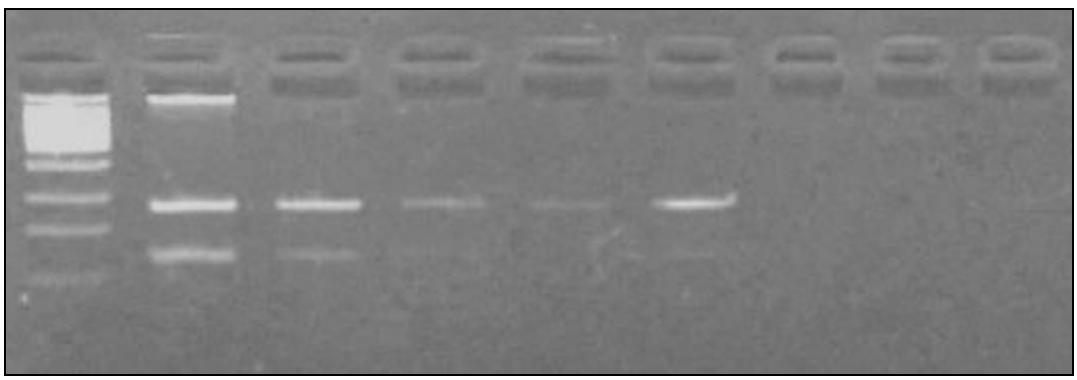

Fig. 3 The PCR amplification of the mtDNA HVR I region – the 188 bp fragment - and the 120 bp from the mtDNA control region V from different aDNA extracts obtained by the Invisorb Forensic Kit (Invitek)-based DNA extraction method:
Line 1: weight molecular marker 100 bp DNA; Line 2: positive control of PCR reaction;
Lines 3, 4: different DNA extracts from the 1I.A sample; Lines 5,6: different DNA extracts from the 2IA sample;
Lines 7, 8: negative controls of different DNA extractions; Line 9: negative control of PCR reaction

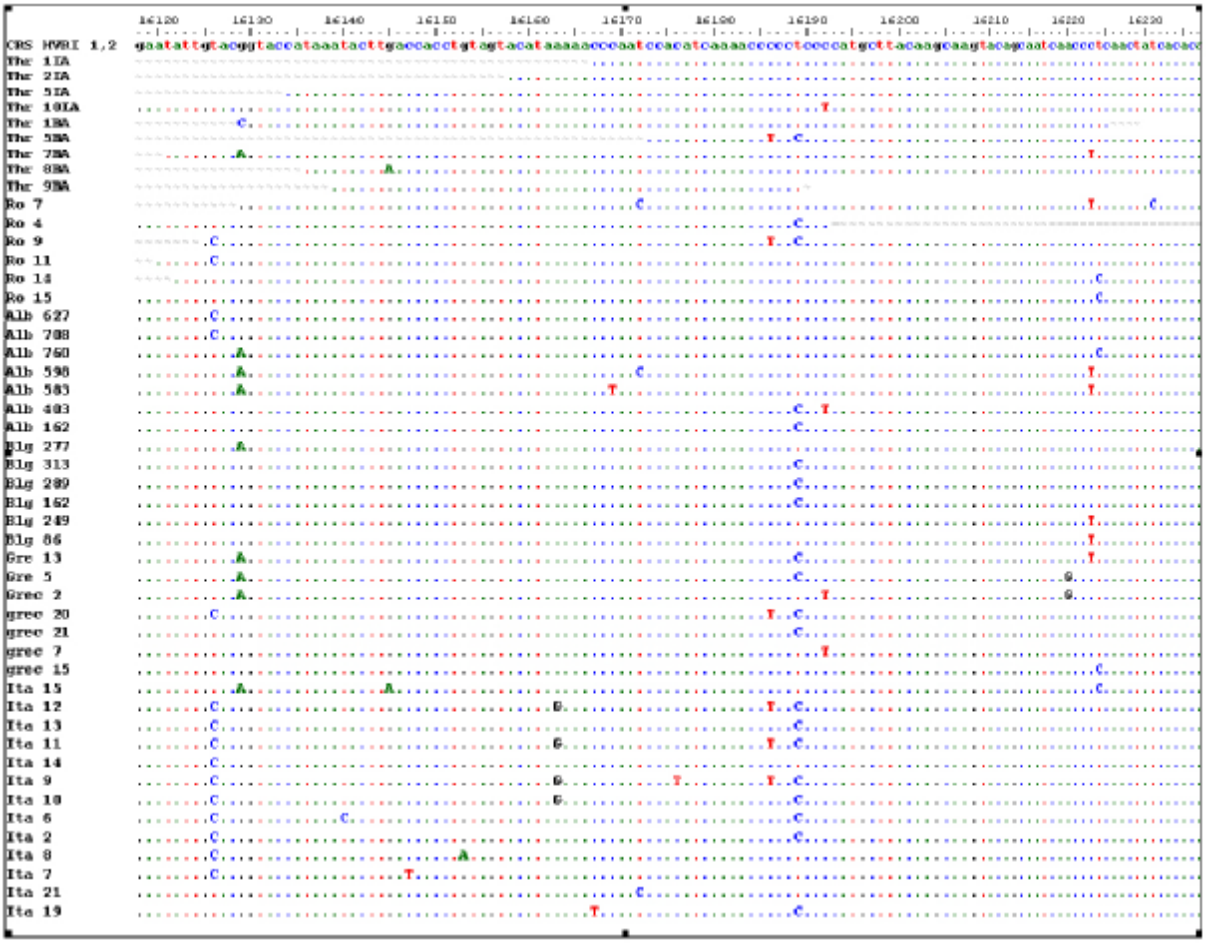

Fig. 4 The comparison of HVR I sequences (the two fragments) of old Thracian populations and 5 European present populations. Thr = Thracian; Ro = Romanian; Alb = Albanian; Blg = Bulgarian; Gre = Greek; Ita = Italian; CRS = Cambridge Reference Sequence; N = unknown nucleotide

First, we have done the alignment of the Thracian mtDNA sequences separately with each sample of 20 sequences from the five modern European populations by using BIOEDIT programme and we noticed that in the mtDNA sequences analysed there are 12 nucleotide positions which gave us some information about the genetic kinship of the five modern European populations with the old Thracian populations (alignments not shown).

Then, from all modern European mtDNA sequences we have chosen only the most informative HVR I sequences, which contain at least one of the 12 informative nucleotide positions, to align them with the mtDNA sequences of the old Thracian populations to show their genetic kinship more relevant (Fig. 4).

The 12 nucleotide positions in which there are common point mutations in comparison with the European mitochondrial sequence CRS, in the sixth populations analysed here are as follows:

1) 16126 np with T→C transition is shown by 10 Italian, 1 Greek, 2 Alban, and 2 Romanian individuals,
2) 16129 np with G→A transition shown by 1 Thracian, 1 Italian, 3 Greek, 1 Bulgarian and 3 Alban individuals,
3) 16145 np with G→A transition shown by 1 Thracian and 1 Italian individuals,

4) 16186 np with C→T transition shown by 1 Thracian, 3 Italian, 1 Greek, 1 Romanian individuals,
5) 16190 np with T→C transition shown by 1 Thracian, 2 Romanian, 8 Italian, 4 Greek, 3 Bulgarian and 2 Alban individuals,
6) 16193 np with C→T transition shown by 1 Thracian, 2 Greek and 1 Alban individuals,
7) 16223 np with C→T transition shown by 1Thracian, 1 Romanian, 1 Greek, 2 Alban and 2 Bulgarian individuals,
8) 16283 np with A→C transversion shown by 1 Thracian and 1 Romanian individuals,
9) 16294 np with C→T transition shown by 1 Romanian, 1 Italian and 1 Albanian individuals,
10) 16311 np with T→C transition shown by 1 Thracian, 1 Romanian, 3 Greek, 4 Italian and 5 Alban individuals,
11) 16356 np with T→C transition shown by 2 Romanian and 1 Bulgarian individuals and
12) 16362 np with T→C transition shown by 2 Thracian, 4 Romanian, 4 Bulgarian and 2 Italian individuals.

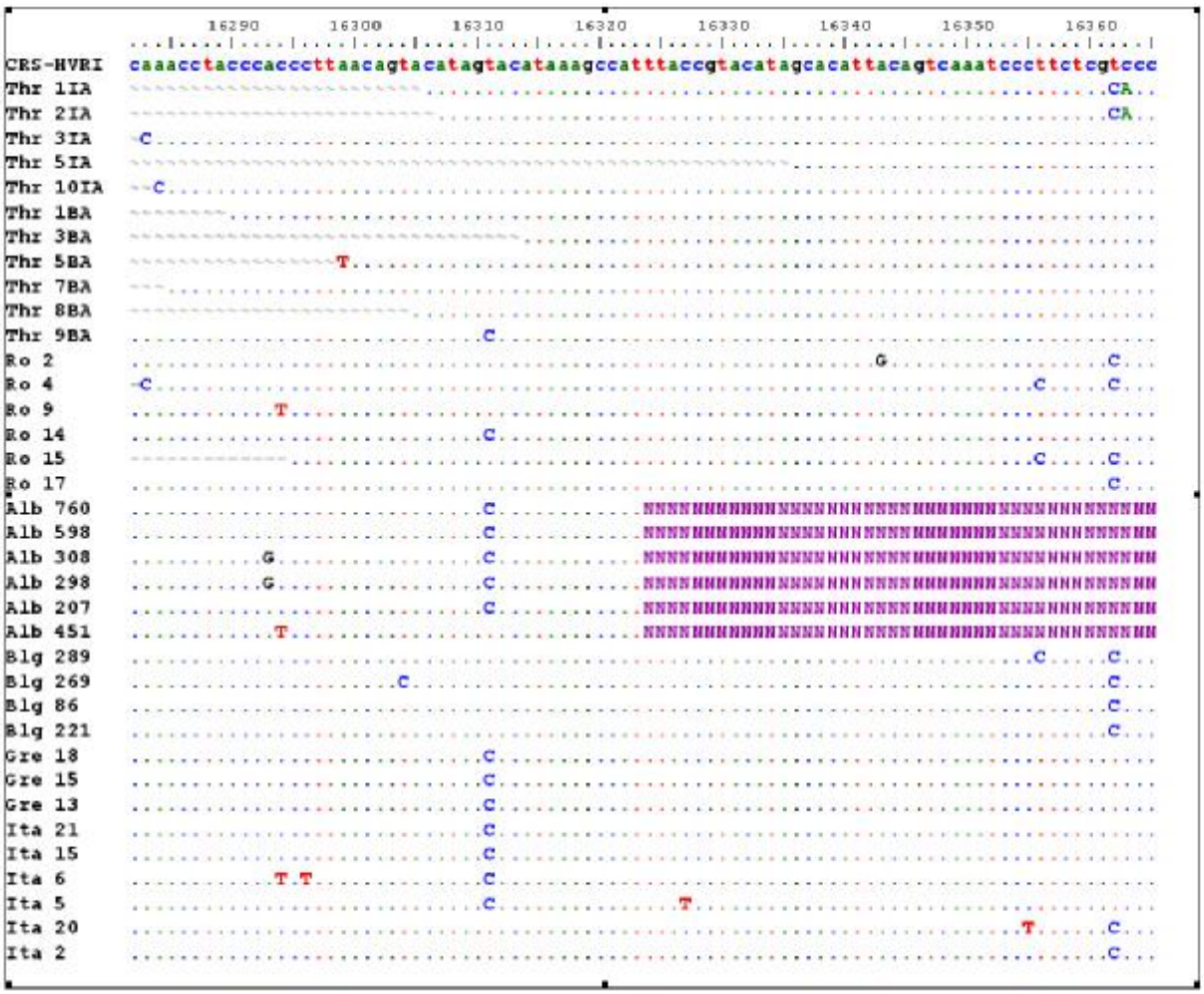

Fig. 4 (continued) The comparison of HVR I sequences (the two fragments) of old Thracian populations and 5 European present populations. Thr = Thracian; Ro = Romanian; Alb = Albanian; Blg = Bulgarian; Gre = Greek; Ita = Italian; CRS = Cambridge Reference Sequence; N = unknown nucleotide

As we can notice in the latter alignment (fig. 4), the Thracian individuals have shown informative point mutations in 7 np, the Romanian, Greek and Alban individuals in 8 np, the Italian individuals in 7 np and the Bulgarian individuals in only 5 np out of the 12 most informative nucleotide positions presented above.

As concerns the frequency of point mutations in the 12 nucleotide positions we have realized that the Italian individuals show the highest mutation frequency with 12.5 %, followed by the Thracian individuals with 8.3 %, the Alban individuals with 7.5 %, the Romanian and Greek individuals with 6.25 % and the Bulgarian individuals with only 4.6 %.

Computing the frequency of common point mutations of the present-day European population with the Thracian population has resulted that the Italian (7.9 %), the Alban (6.3 %) and the Greek (5.8 %) have shown a bias of closer genetic kinship with the Thracian individuals than the Romanian and Bulgarian individuals (only 4.2%).

Conclusions and prospects

In conclusion we believe that our results reflect an evident genetic similarity between the old Thracian individuals analysed up to now in our study and the modern populations from SE of Europe.

So far we can just suppose, that the old Thracian populations would have been able to contribute to the foundation of the Romanian modern genetic pool. More mtDNA sequences from Thracian individuals are needed in order to perform an complex objective statistical analysis.

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