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ORIGINAL ARTICLE

Bulgarians vs the other European populations: a mitochondrial DNA perspective

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Abstract To define the matrilineal relationships between Bulgarians and other European populations, we have evaluated the mitochondrial DNA (mtDNA) variation in a sample of 855 Bulgarian subjects from the mtDNA perspective. The molecular survey was performed by sequencing ~750 bp of the control region, which resulted in 557 different haplotypes, and by a subsequent restriction fragment length polymorphism analysis to confirm haplogroup/subhaplogroup affiliation. The classification was carried out according to the most updated criteria as reported by van Oven and Kayser (*Hum Mutat* 30:386–394, 2009), allowing the identification of 45 mitochondrial clades. The observed pattern of mtDNA variation indicates that the Bulgarian mitochondrial pool is geographically homogeneous across the country, and that is characterized by an overall extremely high frequency of western Eurasian lineages. In the principal component analysis, Bulgarians locate in an intermediate position between Eastern European and Mediterranean populations, which is in agreement with historical events. Thus, while the Mediterranean legacy could be attributed to the Thracians, indigenous people that firstly inhabited the Balkans, the Eastern contribution is likely due to the Proto-Bulgarians originating from the Middle East and to the Slavs migrating from northeast Europe.

Keywords Human mitochondrial DNA · Haplogroup · Bulgarians · Origin of Europeans

Introduction

The territory of Bulgaria has always been a passageway for human migration and an important place for human settlement ever since the first arrival of anatomically modern humans in Europe. Although the gene pool of the contemporary populations of the Old World has been

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primarily shaped by prehistoric demographic events, when characterizing Bulgarian mitochondrial DNA (mtDNA) composition, we cannot disregard more recent migrations. In this context, we should also mention the economic and sociopolitical advancements in the region that sustained the development of the great Thracian civilization at the dawn of recorded human history. The Thracian gene pool was further enriched by the almost simultaneous settlements of Slavs and Proto-Bulgarians. It was initially believed that the number of incoming Proto-Bulgarians was negligible and that they ruled with a limited genetic impact. Today, there is, however, increasing historical and linguistic evidence suggesting that the contribution from Proto-Bulgarians might have been instead substantial. The first settlement of Proto-Bulgarians was possibly located in the foothills of Pamir and Hindu Kush. Afterwards, by "leapfrog" migrations, Proto-Bulgarians would have reached the Caucasus and founded, in the neighboring plains, a state called "Old Great Bulgaria" by Byzantine historians. Subsequently, they moved further westward and colonized two other regions that took the name of Volga Bulgaria and finally Danube Bulgaria (which partially overlaps with the present-day country) [2–4].

Despite the great potentiality of the mtDNA system to identify and associate lineages with geographic origins [5], at present, the information concerning the Bulgarian mtDNA variation is limited to low phylogenetic resolution analyses of a small sample, only considered in a macrogeographic context [6, 7]. Therefore, to improve our knowledge of mtDNA variation in present-day Bulgarians, a very large sample of subjects was analyzed at a high level of haplogroup resolution. The obtained results were then evaluated in the context of West Eurasian populations.

Materials and methods

The sample

The DNA of 855 healthy unrelated subjects of Bulgarian origin was isolated from blood samples using a standard proteinase K digestion followed by phenol–chloroform extraction and ethanol precipitation. Of these, 814 subjects had a precise affiliation to one of the 28 administrative provinces of Bulgaria according to the maternal birthplace.

Genotyping and haplogroup assignment

Control region profiles were determined by sequencing about 750 bp, generally from nucleotide position (np) 16024 to np 210, thus including the entire hypervariable segment I (HVS-I) (nps 16024–16383) and part of HVS-II (nps 57–372). On the basis of differences from the revised Cambridge Reference Sequence (rCRS) [8] that are either diagnostic or associated with specific mtDNA lineages, control region haplotypes were provisionally assigned to haplogroups/subhaplogroups. This affiliation was later confirmed by a hierarchical RFLP survey of haplogroup and subhaplogroup diagnostic markers in the coding region according to the most updated phylogenetic data [1]. The haplotypes presented in this study are available on the EMPOP database (http://www.empop.org) under accession number EMP00450.

Statistical analyses

The genetic diversity within Bulgarians was analyzed by means of chi-square test performed by Excel software implemented by XLSTAT. The same program was used to undertake principal component analysis (PCA) in order to define the matrilineal relationships between Bulgarians and other European and Near Eastern populations. The haplogroups and subhaplogroups included in the PCA are as follows: H* (all H excluding H5), H5, HV0 (including HV0*, HV0a, and V), HV (including HV1 and HV2 without H and HV0), R0a, U1, U2e, U3, U4, U5a, U5b, U6, U7, U8, K, U* (including all U lineages except the aforementioned), J, T1, T2, X, N1 (represented mainly by I), N2 (represented mainly by W), and M. The haplogroups that are different or do not descend from the previously reported clades were labeled as "others" and not considered in the analysis. The Bulgarian dataset employed in the PC analyses also includes a sample of 141 Bulgarian subjects from the literature [7], which was not statistically different from the sample analyzed in this study. The random match probability, a measure of homogeneity in the population, has been calculated as the sum of the squares of the relative frequency of each haplotype [9]. Haplotype (*H*) and nucleotide (π) diversities were obtained using the software DnaSP 5.10 [10], while the average number of nucleotide differences (*M*) was obtained with Arlequin 3.0 [11]. These molecular diversity indices were calculated on 750 base pairs of the control region sequence (from np 16020 to np 16569; from np 1 to np 200), including 850 of the 855 Bulgarian mtDNAs. The remaining five mtDNAs were not included because the reading of their control region sequence did not reach np 200.

Results and discussion

The mtDNA profiles of 855 Bulgarian samples have been determined by direct sequencing of a large portion of the control region (~750 bp). The resulting 557 different haplotypes (Table S1) were classified into 45 mitochondrial haplogroups and subhaplogroups according to the most updated mtDNA phylogeny (Table 1) [1]. As expected, the

Table 1 Frequencies of the major mtDNA haplogroups and sub-haplogroups in Bulgarians

Haplogroup	Subhaplogroup	Number of subjects (%)
H		359 (41.9)
	H*	222 (26.0)
	H1	73 (8.5)
	H3	8 (0.9)
	H5	29 (3.4)
	H6	14 (1.6)
	H8	8 (0.9)
	H11	5 (0.6)
HV0		32 (3.7)
	HV0*	13 (1.5)
	HV0a	6 (0.7)
	V	13 (1.5)
HV (w/o H/HV0)		33 (3.8)
	HV*	30 (3.5)
	HV1	2 (0.2)
	HV2	1 (0.1)
R		7 (0.9)
	R*	3 (0.4)
	R0a	4 (0.5)
U		188 (22.1)
	U1	12 (1.4)
	U2	12 (1.4)
	U3	16 (1.9)
	U4	33 (3.9)
	U5	57 (6.7)
	>U5a	37 (4.4)
	>U5b	20 (2.3)
	U7	4 (0.5)
	U8	4 (0.4)
	>U8a	2 (0.2)
	>U8b	2 (0.2)
	K	50 (5.9)
	>K1	45 (5.3)
	>K2	5 (0.6)
J		67 (7.9)
	J1	60 (7.0)
	>J1b	11 (1.3)
	>J1c	49 (5.7)
	J2	7 (0.9)
	>J2a	4 (0.5)
	>J2b	3 (0.4)
T		91 (10.6)
	T1	37 (4.3)
	T2	54 (6.3)
N		67 (7.9)
	N*	3 (0.4)
	N1	22 (2.6)
	>N1a	6 (0.7)
	>N1b1	6 (0.7)
	>I	10 (1.2)
	N2	24 (2.8)
	>W	24 (2.8)
	X2	18 (2.1)
M		8 (0.9)
	C	2 (0.2)
	D4	3 (0.4)
	M*	1 (0.1)
	M1	1 (0.1)
	Z1	1 (0.1)
L		3 (0.3)
	L0a	2 (0.2)
	L3f	1 (0.1)
Total		855

Bulgarian gene pool is composed almost completely by haplogroups typical of West Eurasian populations [12]. Few exceptions (1.3%) were represented by mtDNAs belonging to East Asian (M, C, D, Z) [13] and African (L0, L3, M1) [14, 15] haplogroups, a common finding in populations of southeastern Europe. As for molecular diversity, considering the above-mentioned 850 mtDNAs (from np 16020 to np 16569; from np 1 to np 200), a total of 520 distinct haplotypes were defined by 204 variable sites. The haplotypes with a frequency >0.005 are listed in Table 2. The probability of random haplotype match of two mtDNAs from Bulgaria was 0.62%; the haplotype and nucleotide diversities were 0.99500±0.00090 and 0.01007±0.00018, respectively, while the average number of nucleotide differences was 7.83692±3.65099.

Haplogroup composition

Super-haplogroup R0

The Bulgarian gene pool incorporates almost the entire spectrum of haplogroups deriving from the basal root of the super-haplogroup R0 (R0a, HV, HV1, HV2, HV0, HV0a, V, and H). Similar to almost all European and Near Eastern populations, the most frequent haplogroup was H (41.9%) [16]. Among Bulgarians, the most represented H subclade was H1 (8.5%), which was most likely involved in the recolonization of Europe after the Last Glacial Maximum and the Younger Dryas glacial relapse (~11.5 kya) [17]. Some other observed subclades of H, such as H5 and H6, are more frequent and typical of the Near East, Caucasus, and Central Asia [17–19]. Their presence in the Bulgarian population (3.4% and 1.6%, respectively) is not surprising considering the history of Bulgaria and its position on the likely entry route of Asian mtDNA lineages into Europe. HV0, a sister clade of H, is scarcely represented and apparently scattered throughout Europe, with a prevalence in the Mediterranean area (including northwest Africa). However, its subclade V is less uncommon and displays a distribution cline radiating from the Iberian Peninsula [20]. In Bulgarians, V accounts for 1.5% of the total population, while the entire HV cluster is observed at a frequency of 3.8%. This latter branch is found at low frequencies in most of its distribution range, which spans from the East Mediterranean to South Europe with a frequency peak in the Near East [21]. Finally, at the root of super-haplogroup R, we found haplogroup R0a with a minor prevalence (0.5%). It harbors almost the same geographic distribution of HV in Eurasia, but its frequency peak is centered in the Arabian Peninsula [22].

Haplogroup U (including K)

Haplogroup U was found to encompass subhaplogroups U1, U2, U3, U4, U5, U7, U8, and K in modern Bulgarians. In this mosaic of lineages, the largest portion belongs to haplogroup U5 (6.7%), which probably arose from the root of cluster U when the first settlers entered the European continent around 45 kya [23]. U5 is represented by both U5a and U5b, (4.4% and 2.3% respectively). One single mtDNA, belonging to haplogroup U5b3, indicates a far link to the Italian refugium area [24]. Haplogroups U1, U2, and U4 are less frequent, occurring at 1.4%, 1.4%, and 3.9%, respectively. U8, a rare haplogroup broadly distributed in Europe, is represented by subhaplogroups U8a and U8b (both 0.2%). U8a is more frequent in west Europe, while U8b occurs beyond Europe, as it is found in the Caucasus, Iran, the Near East, and North Africa. Its subclade K (5.9%) is more widespread and abundant covering the U8a and U8b ranges and even reaching India [25]. The majority of the K mtDNAs belongs to K1 (5.3%), whereas K2 accounts for a minor part of the sample (0.6%). Haplogroups U3 and U7 (1.9% and 0.5%, respectively) are typical of the Near East and of the populations from surrounding areas [16, 26, 27].

Haplogroup JT

Haplogroup J captures 7.9% of the Bulgarian matrilineal diversity and, as expected, is divided into J1 (7%) and J2

Table 2 Most common control region haplotypes detected in Bulgarians

Haplotype ID[a]	Number of subjects (%)	Mutational motif (from np 16020 to np 200)[b]	Haplogroup(s) and frequency (%)
1	46 (5.4)	16519	H (3.3), H1 (1.8), H3 (0.3)
2	12 (1.4)	16304	H5 (1.4)
3	11 (1.3)	16311	HV (0.7), H (0.6)
4	10 (1.2)	16069 16126 16366 16519 73 185 188	J1c (1.2)
5	9 (1.1)	16311 16519	H (0.5), HV (0.4), H1 (0.1), H3 (0.1)
6	9 (1.1)	16126 16163 16186 16189 16294 16519 73 195	T1 (1.1)
7	9 (1.1)	16126 16163 16186 16189 16294 16519 73 152 195	T1 (1.1)
8	9 (1.1)	CRS	H (1.1)
9	7 (0.8)	16224 16311 16519 73 146 152	K1 (0.5), K2 (0.3)
10	7 (0.8)	16356 16519 73 195	U4 (0.8)
11	7 (0.8)	16287 16519 195	H (0.8)
12	6 (0.7)	16298 16311 72	HV0 (0.7)
13	6 (0.7)	16126 16287 16294 16519 73	T2 (0.7)
14	6 (0.7)	16362 16482	H6 (0.7)
15	6 (0.7)	16519 152	H (0.2), H1 (0.2), H3 (0.2)
16	6 (0.7)	16298 72	V (0.6), HV0a (0.1)
17	5 (0.6)	16343 73 150	U3 (0.6)
18	5 (0.6)	16172 16223 16231 16292 16519 73 189 194 195 199	W (0.6)
19	5 (0.6)	16162 16519 73	H (0.6)
20	5 (0.6)	16256 16270 16399 73	U5a (0.6)
21	5 (0.6)	16069 16126 185	J1c (0.6)
22	5 (0.6)	16069 16126 16261 73	J1c (0.6)

[a] The haplotypes are based on the reading of 750 base pairs (from np 16020 to np 16569; from np 1 to np 200) of the control region. Only haplotypes with a frequency >0.005 are listed. Frequencies were calculated in 850 subjects of the total Bulgarian sample (855 subjects). Five subjects were not included because the reading of their control region sequence did not reach np 200

[b] Relative to the revised Cambridge reference sequence [8]

(0.9%). Similarly, haplogroup T includes two subclades: T1 (4.3%) and T2 (6.3%).

Super-haplogroup N

Among the N-rooted clades, N1a, although rare in Bulgaria (0.7%), shows a peculiar phylogeographic separation in N1a* and N1a—the first typical of South Asia/Africa, the second spread in a wide area from Central Asia to Europe. Interestingly, both subclades are equally represented in our sample in agreement with the intermediate geographical position of Bulgaria [28, 29]. N1b1, present at 0.7% in Bulgarians, is also of southwestern Asian origin [30]. The remainder of the haplogroups descending from super-haplogroup N, namely, I, X2, and W, occur at relatively low frequencies (1.2%, 2.1%, and 2.8%, respectively).

Super-haplogroups M and L

The almost exclusive Asian macro-haplogroup M was represented by few instances of East Asian (M*, C, D4, and Z1) [13] and African (M1a) lineages [15]. The HVS-I haplotype of the M* mtDNA matches with one of those previously reported in Bulgarian Romani populations [31]. Few samples have been classified into sub-Saharan African lineages (L0a and L3f, observed in two and one subject, respectively) [14]. These findings are not surprising; in fact, low frequencies of East Asian and African mtDNAs have already been reported in different European populations [26].

Intra-population comparison

In order to evaluate the presence of peculiar trends within our Bulgarian sample, the mtDNA haplogroup distribution in five geographic areas (Sofia area n=154, northeast n=64, northwest n=126, southeast n=215, and southwest Bulgaria n=255; see Table 3) was analyzed with chi-square test. No significant association was observed, indicating that the Bulgarian mtDNA gene pool is rather homogeneous throughout all regions of modern Bulgaria.

Inter-population comparison

In order to visualize the position of the Bulgarian mtDNA profile in the Eurasian and North African context, principal component analysis was performed on 57 European, Asian, and African populations. Since in this preliminary analysis Bulgarians clustered with Europeans, far away from the Southeast Asian countries (not shown), we repeated the PCA on 42 countries from the western part of Eurasia and North Africa. The table

Table 3 Mitochondrial haplogroup frequencies in the different areas of Bulgaria

Haplogroups[a]	Number of subjects					Total
	Sofia area	Northeast	Northwest	Southeast	Southwest	
H	71	24	52	98	95	340
HV	9	4	9	9	21	52
I	2	1	0	1	6	10
J	7	6	9	19	20	61
K	7	4	5	15	17	48
L	0	0	0	3	0	3
M	3	1	2	0	1	7
N	3	1	0	2	8	14
R	3	0	0	1	3	7
T	18	3	18	26	20	85
U[b]	25	11	20	31	47	134
V	2	4	1	2	4	13
W	2	2	5	5	9	23
X	2	3	5	3	4	17
Total	154	64	126	215	255	814

[a] No significant differences among the areas were found in the haplogroup distribution (α=0.05, χ^2 =65.901, p=0.093, df=52)

[b] Haplogroup U does not contain haplogroup K

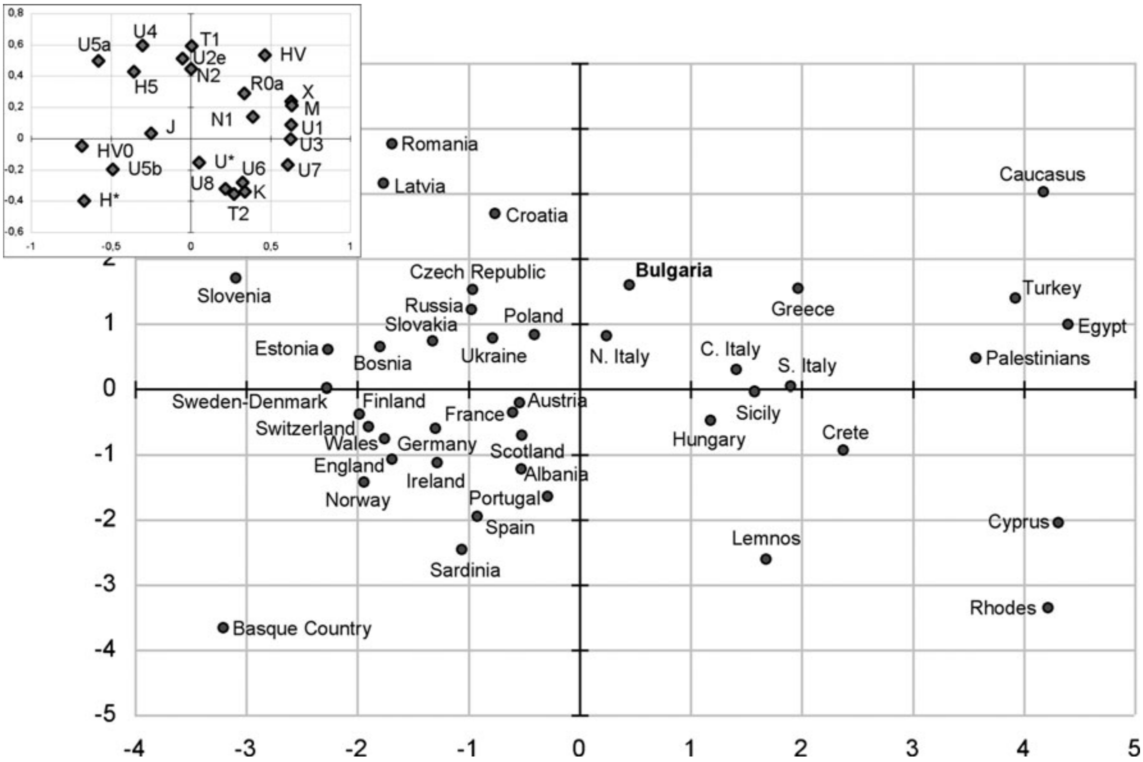

Fig. 1 Principal component analysis. Scatterplot of the first two PCs, retaining in total 32% of the variance: 20% in the first and 12% in the second component. The *upper left* panel shows the contribution of each haplogroup to the first and second PC

illustrating haplogroup frequencies in these populations is reported in the supplementary material (Table S2). Figure 1 shows the plot of the first two principal components where Bulgaria is located at the central part of the graph, being placed in an intermediate position between the Mediterranean countries and the rest of Europe by the first PCA (20% of variance) and in a middle position among east European countries by the second PCA (12% of variance).

Conclusion

With the present study, we provide detailed information on the Bulgarian mtDNA haplogroup diversity. Our analyses show that almost the entire Bulgarian mtDNA pool consists of West Eurasian lineages, some witnessing the Upper Paleolithic (~45–15 kya) colonization of Europe while others marking the Mesolithic expansions (~14.5–10 kya) and the more recent onset of the Neolithic in Europe (from ~8 kya) [23]. It is worth mentioning that historical events are also of crucial importance to explain the current genetic Bulgarian landscape and its localization in the PCA plot, and the central position of Bulgaria probably reflects the admixture of ancient populations of different origin. The Mediterranean contribution, which in the PCA attracts Bulgaria's position far from the other eastern European countries, could be attributed to the indigenous people that inhabited the Balkans and in particular to the great Thracian civilization which reached its apex during the 6th century BC. On the other hand, the Eastern contribution is possibly attributable to the initial Proto-Bulgarians, originating from the Middle East, who merged with the Slavs during the migration towards the Balkans.

Overall, the presented data may serve as a substantial foundation for comparison with other populations and may contribute to the establishment of databases suitable for forensic casework.

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Ethical standards The study was approved by the Bioethics Committee of the University of Pavia, Board minutes of the 5th of October 2010.

Conflicts of interest The authors declare that they have no conflicts of interest.

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