MATERNAL GENETIC PROFILE OF SERBIAN AND MONTENEGRIN POPULATIONS FROM SOUTHEASTERN EUROPE

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Abstract

Significant mtDNA variation in Southeastern Europe (SEE) reflects the turbulent and complex demographic history of the region, influenced by gene flow from various parts of Eurasia and a long history of intermixing. In this study we present the maternal genetic profile of the Serbian and Montenegrin populations based on the high resolution analysis of 258 mtDNAs, 119 samples from Serbia and 139 samples from Montenegro. Besides the evidence of minor gene flow from distant central/northeastern Asia, the majority of haplogroups place these populations in the broader genetic landscape of Southeastern Europe, close to their neighbors.

Key words: *mtDNA, Serbia, Montenegro, Southeastern Europe, haplogroup*

Introduction

The region of Southeastern Europe (SEE) has been inhabited since the Middle Paleolithic, with a key role in the Upper Paleolithic recolonization of Europe and the Neolithic spread of agriculture (Forenbaher & Miracle, 2005). Based on the analysis of uniparental markers (mitochondrial DNA and Y chromosome), the current patterns of genetic variation can be utilized to gain insights into past population processes, migrations and other demographic events and in the last two decades significant results have been published regarding peopling of the world and specific genetic structure and variation of different populations (Karmin et al., 2015; Nielsen et al., 2017). Results of mitochondrial DNA variation of this region published so far reflect the turbulent and complex demographic history of SEE, influenced by gene flow from various parts of Eurasia and a long history of intermixing (Malyarchuk et al., 2003; Cvjetan et al., 2004; Bosch et al., 2006; Peričić et al., 2005; Šarac et al. 2014; Kovačević et al. 2014, Davidović et al. 2015). The general aim of this study is to enrich the current mtDNA database on Slavic-speaking, Southeast European populations that share the same geographic area, are of the same religious background and have a long history of cohabitation in this region. The specific aim is to present a detailed view of the mtDNA landscape of two SEE populations, Serbians and Montenegrins, in order to analyze more closely how they fit within the wider SEE context.

Table 1. Primers used for the amplification of the mtDNA control (HVS) region

mtDNA fragment		name	primer sequence	ann. temp (°C)	product bp.	
HVS-I	F	V3	5'-15806-GCATCCGTACTATACTTCACAACAATCC	52	739	internal primers
	R	HVS-I R	5'-16545-AACGTGTGGGCTATTTAGGC			
	F	mitF	5'-15879-AATGGGCCTGTCCTTGTAG	56	666	
	R	HVS-I R	5'-16545-AACGTGTGGGCTATTTAGGC	56		
HVS-II	F	16495	5'-16495-CGACATCTGGTTCCTACTTC	52	445	F(16495)
	R	370	5'-370-GGTTCTTTGTTTTTGGGGTT			
	F	H	5'-15975-CTCCACCATTAGCACCCAAAG			
internal primers	F	A	5'-15909-ACACCAGTCTTGTAAACCGG	50		
	R	F	5'-16420-TGATTTCACGGAGGATGGTGG			

Materials and methods

Sample

Our sample consists of 258 unrelated individuals from two SEE populations; 119 samples from Serbia, published previously (Cvjetan et al. 2004) and presented here in higher resolution and 139 samples from Montenegro that are reported here for the first time. Geographic locations of the SEE and position of our sampled populations within it are depicted in Figure 1. Blood samples were collected from healthy adults after obtaining informed consent and all examinees completed an extensive questionnaire with genealogical information that allowed exclusion of potentially related individuals up to the grandparent level. The DNA was extracted from whole blood according to the standard 'salting out' method (Miller et al., 1988) at the Institute for Anthropological Research in Zagreb, Croatia. All further laboratory analyses were performed at the Estonian Biocentre and Department for Evolutionary Biology, Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia.

Methods

The hypervariable segment I (HVS-I) of the control region of mtDNA (nucleotide positions 16000 – 16400) was PCR amplified by Biometra Thermocycler and sequenced on ABIPrism 3130xl Genetic Analyser (Applied Biosystem, Foster City, CA, USA), using the Big Dye Terminator kit (Applied Biosystems, Warrington, UK). Sequences of primers used in DNA amplification are listed in Table 1. Haplogroup and subhaplogroup detection was based on single nucleotide polymorphisms (SNPs) specific for main Eurasian lineages, with a combined usage of restriction fragment length polymorphism method (RFLP method) or direct Sanger sequencing methodology. When using the RFLP method, the PCR products were incubated with restriction enzyme and fragments were then separated on agarose gels of varying concentrations (2.0 - 3.5%), depending on differences in the length of DNA fragments, by staining with ethidium bromide. The restriction pattern was visualized using an UV transilluminator. List of enzymes used in RFLP analysis can be seen in Table 2.

Figure 1. Geographic location of the sampled populations (left), position of sampled populations within the wider European region (right)

In cases when the RLFP method was unavailable due to the lack of restriction site for an enzyme, the mtDNA segment of interest was amplified by PCR and sequenced. In order to enable comparison of our results with the ones from other mtDNA studies, the HVS-I sequences were aligned and analyzed according to the revised Cambridge Reference Sequence (rCRS; NC_012920) (Andrews et al.,

Table 2. Polymorphic sites, diagnostic for specific mtDNA haplogroups and methods for their detection (+ denotes presence of a restriction site; - denotes absence of a restriction site)

Haplogroup	np	type		enzyme
H	7028CT	RFLP	-	7025 AluI
H1	3010GA	RFLP	-	Bsh1236
H2*	1438	RFLP	+	BseMII
H2a	4769GA	RFLP	+	4769 AluI
H3	6776TC	sequencing	×	
H4	5004TC	RFLP	-	5003 DdeI
H5	456CT	sequencing	×	
H6	16482AG	RFLP	+	16478 DdeI
H6	239TC	sequencing	×	
H7	4793AG	RFLP	+	4793 BsuRI
H8	13101AC	RFLP	+	13100 MspI
H11	8448TC	RFLP	-	8446 SspI
H12	14552G	sequencing		
H13	14872T	sequencing		
H15	6253	sequencing		
HVO, V	15904CT	RFLP	+	15904 Tru1I /MseI
I	10034TC	RFLP	+	10032 AluI
J	13708GA	RFLP	-	13704 MvaI (BstOI)
N1a,L	10398AG	RFLP	+	10394 DdeI /HpyF3I
N	1719	RFLP	-	1715 HpyF3I (DdeI)
J2	7476CT	RFLP	-	7474 AluI
J1b	8269	sequencing	X	
J1	3010GA	RFLP	-	3007 Bsh1236I
J2a	10499	sequencing	x	
J2b	5633CT	RFLP, sequencing	+	5633 AluI
K	9055GA	RFLP	-	9052 HaeII
K1	1189TC	sequencing	×	
K1a	497	sequencing	×	
K1b	5913GA	sequencing	×	
K1c	497CT	sequencing	×	
K2a	4561TC	sequencing	×	
T	13368GA	RFLP	+	13366 BamHI
T1	12633CT	RFLP	-	12629 Eco47I
T2	11812AG	sequencing	×	
T2b	930GA	sequencing	×	
U	12308AG	RFLP	+	12308 HinfI
U1a	4991AG	RFLP	-	4990 AluI
U2e	15907AG	RFLP	+	15907 RsaI
U4	4646TC	RFLP	+	4643 RsaI
U5a1	14793	sequencing	x	
U5b1	5656AG	sequencing	×	
U5b	7768AG	sequencing	×	
V	4580GA	RFLP	-	4577 NlaIII
W	8994GA	RFLP	-	8994 BsuRI
W1	7864	sequencing	x	
X	14470TC	RFLP	+	14465 BstI107

1999) by using ChromasPro software (Technelysium Pty Ltd, Tewantin QLD, Australia). mtDNA tree Build 17, updated February 18, 2016, was consulted while defining haplogroup affiliations (www.phylotree.org) (Van Oven & Kayser, 2009). Principal Component Analysis (PCA) was performed as a visual representation of the differences between the populations based on mtDNA subhaplogroup frequencies, using the free software POPSTR (http://harpending.humanevo.utah.edu/popstr/).

Results and Discussion

The obtained results show that the Serbian and Montenegrin mtDNA diversity fits within the wider SEE maternal genetic landscape, as expected (Cvjetan et al., 2004; Bosch et al., 2006, Šarac et al. 2014, Kovačević et al. 2014; Davidović et al. 2015). However, in spite of the geographical proximity of the SEE populations (Fig. 1), certain differences can be observed in mtDNA haplogroup composition and variation. Haplogroups and their frequencies in each of the sampled populations are presented in Figure 2 and Tables 3 and 4. A high degree of haplotype diversity has been established and, in total, 13 major haplogroups, 49 sublineages and 160 haplotypes have been detected in 258 analyzed samples. Less than 2% of the samples can be attributed to haplogroups of non-European lineage, which is their typical portion in the European mtDNA gene pool (Šarac et al. 2014). The most dominant European clade, H haplogroup (hg), is found in 44.3% of the total sample, followed by hgs U (17.7%) and J (8.8%).

Our results have confirmed haplogroup H as a prevalent clade in the sample with a frequency of 40.34% among the Serbians and 48.20% among the Montenegrins. Macrohaplogroup H has been in the focus of human genetic diversity studies for almost 15 years, with an estimated coalescence time of ~20,000 ya. Examining the spatial distribution of H lineages and other features associated with its evolutionary history has led to the proposal that this clade was involved in a post-glacial re-expansion of populations from southwestern Europe to the rest of the continent (Hernandez et al. 2017). The dominance of this lineage among the Serbians and Montenegrins is also in line with previous studies of hg H variation in the SEE region (Cvjetan et al., 2004; Peričić et al., 2005; Loogvali et al., 2004, Alvarez-Iglesias et al., 2009, Šarac et al. 2014, Kovačević et al. 2014), with a rich inner diversification (subhaplogroups H*, H1, H2, H3, H4, H5, H6, H7, H11, H12, H13, H14 have been found in the total sample). However, paraphyletic cluster H* and hgs H1, H2 and H5 account for more than 60% of the H portion among Serbians and Montenegrins. These are typical H haplogroup results for SEE.

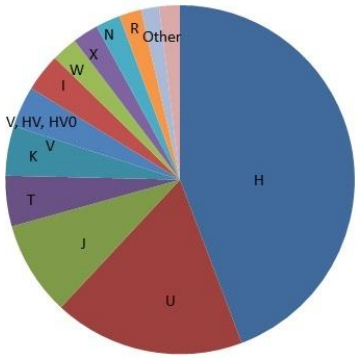

Figure 2. mtDNA frequencies of the total sample (N=258)

Haplogroup U is the second most frequent haplogroup in Europe and in our sample as well (21% among the Serbians and 14.4% among the Montenegrins) and it is considered to be the oldest European hg, especially its U5 sublineage with an estimated coalescence time of about 36,000 ya (Soares et al., 2010; Malyarchuk et al. 2010). In our sample U5 also represents the dominant U sublineage, harboring almost half of all U individuals. Interestingly, the Serbian population harbors significantly more U4 individuals (6.7%) than the Montenegrin population (2.2%). U4 is in general the second largest U subhg in Europe and, similar to U5, it shows "molecular signals" for late glacial and post-glacial expansion (Malyarchuk et al. 2010). Other sublineages, such as U1, U2e, U3, U6, U7 and U8 are all present in our sample, however in a much smaller portion.

Haplogroup J started to spread from the Near East into Europe immediately after the peak of the last glaciation, ~19 000 ya, together with haplogroup T, and its major expansion in Europe followed in the Late Glacial period, ~16–12 000 ya (Pala et al. 2012). Haplogroup J is the third most prevalent

Table 3. mtDNA variation in the serbian and montenegrin population

HG	HAPLOTYPE	SRB	MNE	HG	HAPLOTYPE	SRB	MNE
A8	223-242-290-319-357	1		H5	304	4	5
D4	174-362		3	H5a1e	166C-304		1
H*	168	1		H5	294-304		1
H*	189	3	4	H5	304-311		1
H*	192	1	1	H5a1	93-304	1	
H*	209	1		H6	362	1	1
H*	212		1	H7	129	1	
H*	299		1	H7	189		1
H*	311	3	2	H7	311	1	
H*	129-189		1	H7c1	93-234-265		1
H*	162-278-292-368		1	H7c1	93-265	2	
H*	162-368	1		H7	CRS	3	1
H*	241-311	1		I1a1a3	129-172-223-311-319-391	1	
H*	256-319		1	I	129-172-223-311-391	2	
H*	291-390	1		I4a1	129-223-304		1
H*	311-355-376		1	I	129G-172-223-311		1
H*	51-312	1		I1a1a3	80-223-311-319-391	1	
H*	86-153		1	J1b1a1	69-126-145-172-222-261		1
H*	93-129	1		J1b1a1	69-126-145-172-261		1
H*	93-212-222		1	J1c	69-126-193-362	1	
H*	CRS	4	4	J1c	69-126-366	2	
H1	188	1	1	J1c	69-93-126		3
H1	189		1	J1c	69-93-126-261		1
H1	209	1	1	J1c	69-93-126-433		1
H1	311	1		J1c1	69-126	1	2
H1	92-189-209	1		J1c3f	63-69-126-348		2
H1	CRS		3	J1c2e	69-126-366		3
H11	311		2	J2a	69-126-145-189-231-261	2	
H11a	169-293-311		1	J2a	69-126-145-231-261-291	1	
H11a	278-293-311	1		J2a	69-126-189-231-261	1	
H11a	293-311		1	J2b1	69-126-193		1
H11a	92-261-293-311		1	K	224-311-354	1	
H11a	92-293-311		3	K1a	129-224-301-311		1
H12a	287		3	K1a	223-224-234-304-311	1	
H12a	287-311		3	K1a	224-304-311		1
H13	188	1		K1a	224-311-354	2	
H13	304	1		K1a	224-311-360		1
H13	48-261-270	1		K1a	93-224-311		1
H13	CRS	1		K1a	93-224-311-354		1
H13a2	148-188-256-319		2	K1a	93-224-311-362		1
H13a2	148-256-319		1	K1b	224-311	1	1
H14	256-352-380		1	N	86-172-187-189-217-223	2	
H1b	189-356	1		N1b	145-176CA-241-223-390	1	
H1b	189-356-362	1		N1b	145-176-CG-223-390	1	
H1b	189-356-362		3	N1b	145-176G-223-244		1
H2*	287-311	1		R	311	1	
H2a	311	2	2	R	CRS	2	
H2a	CRS	2		R0	126-168-266-304-362	1	1
H2a1	354		2	R0	217-243-261		2
H2a1	140-354		1	HV	311	1	3
H2a1	249-354		1	HV0	126-298-311	1	
H3	311		2	HV0	298-311	1	
H4	CRS		1	T1a	126-163-170-186-189-294		2

HG	HAPLOTYPE	SRB	MNE	HG	HAPLOTYPE	SRB	MNE
T1a	126-163-186-189-261-294	1		U5a1	256-270		1
T1a	126-163-186-189-294	3		U5a1	75-129-270-256-399	1	
T1a	126-163-186-189-294-390		1	U5a1	75-256-270-353		1
T2	126-294		1	U5a1	93-172-192-256-270-399	1	
T2	126-294-296-362	1		U5b1	189-213-270	1	
T2b	126-243-294-296-304	1		U5b1	93-189-270	1	
T2b	126-243-294-296-304		1	U5b1	93-189-270-301-319	1	
T2b	126-294-296-304		1	U5b1b1	129-144-189-270		1
U	254		1	U5b1b1	144-189-270	3	1
U*	CRS	1		U6	172-189-234-270-311	1	
U1a	126-189-249-353-360-362		2	U6b	172-189-234-311		2
U2e	51-129G-189-270-362		1	U7	172-318T	1	
U2e	51-129G-256-311-362		1	U7	309-318T	1	
U2e	51-129GC-189-362-319	1		U8a	146-180-342		1
U2e	51-129GC-311-362	1		V	298	2	5
U3	343	1		V	162-298	1	
U4	356	3		V	216-261-298	1	1
U4	134-356-362	2		W1	223-292-295-324	3	
U4	179-356		3	W1	223-292-311		1
U4	356-362	2		W1	66-129-145-223-292		1
U4	51-179-356-362	1		W3	223-292	1	
U5	192-270-304		1	X*	129-173-189-223-266-274-278-390		1
U5	93-189-270		2	X2*	189-192-223-278-292		1
U5a	192-256-270	1		X2*	189-278-316	2	
U5a	256-270	1		X2b	189-223-278		1
U5a	66-192-256-270	1		X2d	189-223-227-278-287-290-362		1
U5a1	192-217-256-270-399		1				
U5a1	192-256-270-291		1		**TOTAL**	**118**	**139**

lineage in the general sample and individually among Serbians (6.7%) and Montenegrins (10.8%) as well. Kovačević et al. (2014) stated in their paper that sub-hg J1 is the most frequent and widespread in all studied Western Balkan populations and it is also well diversified in this sample. Interestingly, it is significantly more diversified in the Montenegrin population, in comparison with the Serbian one.

Other typically European haplogroups (T, K, I, V, HV, X, W) are represented in the general sample with a similar frequency as reported previously for this region of Europe, without significant differences between the Serbians and Montenegrins (Šarac et al. 2014; Kovačević et al. 2014; Davidović et al. 2015). Non-European haplogroups present in the sample are hg A and D, more specifically A8 and D4 subclades. Haplogroup A is believed to have arisen in Asia some 30,000–50,000 BP and is most abundant among Native Americans and East Asians, while haplogroup D is the second most common haplogroup in all northern Asian populations (20%) and also very common in eastern/central Asia and America (Derenko et al. 2010, Fedorova et al. 2013). The presence of these lineages indicates minor signals of long-distance migrations (most probably single events from central/northeastern Asia) which have left a trace in the genetic heritage of these SEE populations.

In order to place the Serbian and Montenegrin population in a wider SEE context, we compared them to Albanians, Macedonians, Greeks, Bosnians, Herzegovinians, Bulgarians, Croatians, and Romanians. In order to visualize these relations, a PCA plot has been constructed based on haplogroup frequencies of the named SEE populations (Fig. 3). The reference data used for the comparative analyses are presented in Table 2. Genetic profiles of the populations under study resemble those of their geographical neighbors and are clustered among them. The most prominent outlier is Greece, which is also the southernmost population in the comparative sample (located geographically in

Table 4. mtDNA haplogroup frequencies in sampled populations and other see populations used for comparison in the pca plot

POP	N	REFERENCE	H	J	T	K	U*	U1	U2	U3	U4	U5
Greece	25	Bosch et al., 2006	48.00	8.00	16.00	0.00	0.00	0.00	0.00	0.00	0.00	4.00
Albania	42	Bosch et al., 2006	50.00	4.80	7.20	4.80	0.00	2.40	0.00	0.00	4.80	7.00
Macedonia	37	Bosch et al., 2006	45.90	5.40	16.20	5.40	5.40	2.70	0.00	0.00	5.40	2.70
Bulgaria	855	Karachanak et al., 2012	41.90	7.90	10.60	5.90	0.00	1.40	1.40	1.90	3.90	6.70
Romania	59	Bosch et al., 2006	42.40	8.50	6.80	11.9	3.40	0.00	1.70	0.00	1.70	5.10
Croatia	488	Šarac et al., 2014	46.64	9.76	6.49	4.25	0.00	0.76	1.95	1.44	2.59	10.04
Bosnia	239	Šarac et al., 2014	42.68	7.53	3.35	6.69	0.00	0.42	0.84	0.84	4.18	8.79
Herzegovina	130	Šarac et al., 2014	43.08	8.46	6.15	9.23	0.00	1.54	2.31	0.00	4.62	4.62
Slovenia	97	Šarac et al., 2014	38.14	14.43	15.46	5.15	0.00	1.03	4.12	1.03	1.03	13.40
Serbia	119	Cvjetan et al., 2004; this study	40.34	6.72	5.04	4.20	0.84	0.00	1.68	0.84	6.72	9.24
Montenegro	139	this study	48.20	10.79	4.32	5.04	0.72	1.44	1.44	0.00	2.16	6.47
Total	2311											

POP	N	REFERENCE	U6	U7	U8	R	N	I	W	X	V,HV HV0	Other
Greece	25	Bosch et al., 2006	0.00	0.00	0.00	0.00	4.00	4.00	4.00	8.00	0.00	8.00
Albania	42	Bosch et al., 2006	0.00	0.00	0.00	0.00	9.50	0.00	2.40	0.00	7.20	0.00
Macedonia	37	Bosch et al., 2006	0.00	0.00	0.00	0.00	2.70	0.00	2.70	0.00	5.40	0.00
Bulgaria	855	Karachanak et al., 2012	0.00	0.50	0.40	4.70	1.75	1.20	2.80	2.10	3.72	1.20
Romania	59	Bosch et al., 2006	0.00	0.00	0.00	3.40	1.70	0.00	0.00	3.40	3.40	0.00
Croatia	488	Šarac et al., 2014	0.16	0.24	0.16	0.00	1.05	1.91	2.26	1.96	7.80	0.16
Bosnia	239	Šarac et al., 2014	0.42	1.67	0.42	2.51	0.42	2.51	3.77	2.09	10.88	0.00
Herzegovina	130	Šarac et al., 2014	0.00	0.00	0.00	0.00	1.54	0.77	2.31	0.77	13.08	1.54
Slovenia	97	Šarac et al., 2014	0.00	0.00	1.03	0.00	0.00	0.00	1.03	1.03	4.12	0.00
Serbia	119	Cvjetan et al., 2004; this study	0.00	1.68	0.00	3.36	3.36	3.36	3.36	1.68	5.88	1.68
Montenegro	139	this study	1.44	0.00	0.72	0.00	0.72	1.44	1.44	2.88	8.63	2.16
Total	2311											

Southern, not Southeastern Europe) and under the influence of maritime migrations and gene flow from other regions.

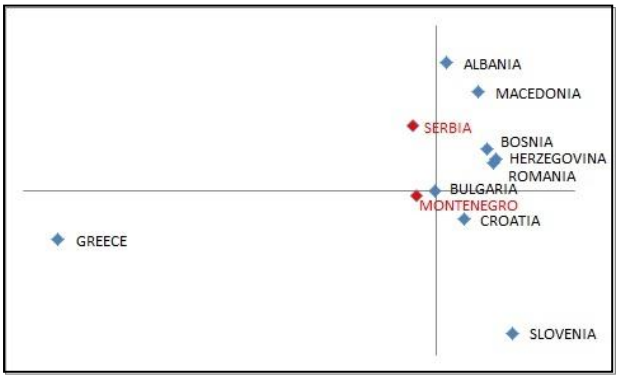

Figure 2. PCA plot visualization of mtDNA frequencies in sampled populations and other neighboring SEE populations

The outlying position of Slovenia also supports the previously proposed hypothesis that populations of the northern part of SEE fit better into the central/eastern European context than into the southern European one (Malyarchuk et al., 2003; Šarac et al. 2014).

Conclusions

This study shows that the maternal genetic heritage of Serbians and Montenegrins is in concordance with the general European mtDNA gene pool and that of the neighboring populations. We can conclude that, in spite of cultural and ethnic diversity of SEE populations, this region consists mainly of Slavic-speaking populations that share the

same geographic background and have a long history of cohabitation in this region, which is why they are genetically quite homogenous.

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