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Abstract

Ancient (proto-) Bulgarians have long been thought to as a Turkic population. However, evidence found in the past three decades show that this is not the case. Until now, this evidence does not include ancient mitochondrial DNA (mtDNA) analysis. In order to fill this void, we have collected human remains from the VIII-X century AD located in three necropolises in Bulgaria: Nojarevo (Silistra region) and Monastery of Mostich (Shumen region), both in Northeast Bulgaria and Tuhovishte (Satovcha region) in Southwest Bulgaria. The phylogenetic analysis of 13 ancient DNA samples (extracted from teeth) identified 12 independent haplotypes, which we further classified into mtDNA haplogroups found in present-day European and Western Eurasian populations. Our results suggest a Western Eurasian matrilineal origin for proto-Bulgarians as well as a genetic similarity between proto- and modern Bulgarians. Our future work will provide additional data which will further clarify proto-Bulgarian origins; thereby adding new clues to current understanding of European genetic evolution.

The development of anthropogenetics and paleogenetics and the increase of their role in evolutionary science have given rise to a new scientific field of ancient DNA research. Technological improvements now allow the retrieval of mtDNA from museum specimens, archaeological finds, and fossil remains. By sequencing mitochondrial hypervariable fragments of ancient DNA extracted from skeletal remains, researchers can classify mtDNA into maternal lines according to their sequence polymorphisms, thereby creating chronologies that link contemporary humans with their ancestors (Adachia et al. 2004; Forster 2004; Yao and Zhang

2000; Yonggang and Yaping 2003). In this study, we used ancient mtDNA to investigate the origins of proto-Bulgarians.

Many studies have focused on the origins of ancient (proto-) Bulgarians. This interest is most likely related to the fact that Danubian Bulgaria, the proto-Bulgarian state created in the seventh century AD is the only ancient state in Europe that has retained its name to the present day. The ancient Bulgarian state was officially recognized by the Eastern Roman Empire (Byzantium) in 681, after Kan Asparuh led its army to victory over the 80 000 army of the Eastern Roman Empire (Byzantium) in 680. At that time Bulgaria extended to the Balkan Mountains.

One concept of the origin of proto-Bulgarians defines them as a Turkic population, mostly referred to as Hun-Tatars (Huns Mongols). Tatars are the Russian designation for the Mongols—descendants of Genghis Khan, who invaded Russia in the thirteenth century. This hypothesis was first presented in *Dějiny národa bulharského* (History of the Bulgarians) by a Czech historian, diplomat, and Slavicist, who worked as a politician in Bulgaria from 1879 to 1884 (see Jireček 1878 and Jireček 1876 for English and German translations). This idea was followed by a prominent Bulgarian medievalist (Slatarski 1918; Zlatarski 1914, 1918, 1970), and still has followers to this day; some of whom claim that the small Bulgarian horde was "submerged" in the Slavic demic sea.

However, researchers such as Peter Koledarov, Peter Dobrev, and Georgi Bakalov reject the idea of a Hun-Tatar (Turkic) origin for proto-Bulgarians (Dobrev 1991, 1998, 2005; Fol et al. 2000), and recently the number of those who agree with them has been increasing (e.g., Daskalov 2011; Haefs 2009; Stamatov 1997). Their rejection of a Hun-Tatar origin is based on archeo-anthropological, historical, linguistic, and ethnographic evidence, which has been increasing over the past three decades.

Such research has shown that, following the second century, proto-Bulgarians created three countries in Europe: Danubian Bulgaria, Volga-Kama Bulgaria, and Old Great Bulgaria in northern Caucasus. They also built town-fortresses, organized powerful armies, and developed civilizations, economies, and art. Leading Turkologists have also presented evidence that the language of proto-Bulgarians does not reflect the Turkic linguistic family; instead it gravitates toward the Pamir languages of the East Iranian group, which belong to the Indo-European branch of languages (Bazin 1974; Manchen-Helfen 1973; Menges 1968; Pritsak 1955). Furthermore, writings from ancient Greek, old German, old Khazar, and Proto-Bulgarian authors suggest that proto-Bulgarians were a numerous people (Beshevliev 1993; Daskalov 2011; Dujchev 1963; Petrov and Gjuzelev 1979), comprising 32–60% of the population of Danubian Bulgaria (Dimitrov 2005; Rashev 1993). As history lacks examples of advanced, developed populations, such as proto-Bulgarians, being assimilated by tribes that are at an early stage of social development, like the Balkan Slavic tribes, it is unlikely that proto-Bulgarians were subsumed by such a group.

To date, analysis of ancient mtDNA from remains found in Bulgaria is missing from the literature. Thus, we report the first data from mitochondrial phylogenetic analysis of ancient DNA retrieved from human remains found in Bulgarian lands. We describe the mtDNA composition found in our samples and discuss the obtained data from genetic, anthropological and historical point of view in order to unravel the origin of ancient proto-Bulgarian populations.

Materials and Methods

In order to minimize possible founder effects, we have analyzed human skeletal remains found in different Bulgarian lands and dating to different periods of the first Bulgarian state - Danubian

Bulgaria (VIII-X century AD). The Danubian Bulgaria population consisted primarily of proto-Bulgarian and Slavic tribes who occupied areas inhabited in antiquity by Thracian populations. The proto-Bulgarians practiced typical burial traditions, whereas the Slavs practiced cremation (Jordanov and Timeva 2010; Rashev 2008; Rashev et al. 1986, 1987, 1988, 1989). Based on this and on historical and anthropological data, the analyzed remains are considered as proto-Bulgarian.

Specimens (teeth) were collected from graves in three necropolises: the Monastery of Mostich (Shumen region) and Nojarevo (Silistra region) in Northeast Bulgaria; Tuhovishte (Satovcha region) in Southwest Bulgaria (Figure 1). Table 1 provides descriptions of the analyzed samples.

The three necropolises were first found and investigated in the mid-twentieth century. The first necropolis, the Monastery of Mostich, is in the outer southeastern area of Veliki Preslav, Shumen region. Its monastic identification is based on the burial inscription for the *icirgu-boilas* Mostich, a former military and administrative officer who later became a monk. He was reburied in a tomb in the north wall of the church. Three other tombs with buried and reburied monks were found in the south wall of the church. These tombs show evidence of burial practices typical for medieval Bulgarian monasteries. Another bipartite brick tomb, discovered in the western porch of the church, was affiliated to the noble monastic founder (George, the Bulgarian *synkellos*) and his closest relatives (Popkonstantinov and Kostova 2010, 2011, 2012, 2013).

Nojarevo, the second necropolis, is an early medieval necropolis characterized as pagan and biritual. Most graves are inhumation graves with specific corpse positioning and often with artificially deformed skulls and bones (Jordanov and Timeva 2010; Rashev 2008; Rashev et al. 1986, 1987, 1988, 1989).

The third necropolis is near the village of Tuhovishte in the southwestern Rhodope Mountains, Chech region. The stone graves are mostly inhumation, though some are cremation (Serafimova 1981).

The traditional methodology consisting in three fundamental steps was followed: i) PCR amplification of several short and overlapping target fragments to recover larger HVS I regions; ii) Production and sequencing of several clones for each amplified fragment; and iii) Alignment and comparison of sequences from different clones and different overlapping fragments to reconstruct the final consensus sequence of the entire region of interest (Rizzi et al. 2012).

All methods for preparing, extraction, and analyses of ancient mtDNA followed strict protocols (Hofreiter, Jaenicke et al. 2001; Paabo et al. 2004). The teeth were cleaned and powdered using a rotary tool, and mtDNA was extracted using a silica-based protocol (Caramelli et al. 2008; Hoss and Paabo 1993).

We analyzed sequences from hypervariable segment I (HVS-I) because most mtDNA variations belong to this region; it is also the region most commonly used for tracing human origins. Following the standard procedures (Caramelli et al. 2008; Pilli et al. 2013) , we used AmpliTaq Gold® *Applied Biosystems*® to perform several steps of amplification and quantification of overlapping fragments covering 360 bp from HVS-I. The HVS-I was retrieved in three overlapping fragments (L15995-H16132, L16107-H16261 and L16247-H16402). For each step, the quality and quantity of fragments were checked by agarose gel electrophoresis. Amplified fragments were cloned using specific competent cells (*Escherichia coli*) and the TOPO TA Cloning® Kit*, Life technologies* ™. Recombinant colonies were screened by PCR and purified by Microcon®, *EMD Millipore*® PCR purification. The products were Sanger sequenced using the BigDye® Terminator Kit, *Applied Biosystems*®.

Variation between samples was evaluated using *t* tests, and sample sequences from the separate clones of different amplicons were aligned and compared. Nucleotide changes occurring at particular positions in only one or two clones were considered amplification- or cloning-procedure errors. However, substitutions observed in a majority of clones were considered real mutations and were reported in the final consensus sequences. These ancient mtDNA variations were determined by aligning mtDNA sequences to the revised Cambridge reference sequence (rCRS). HVS-I haplotypes were classified into possible haplogroups and sub-haplogroups using HaploGrep (Andrews et al. 1999; Hofreiter, Serre et al. 2001; Van Oven and Kayser 2009; Kloss-Brandstaetter et al. 2011).

The obtained haplogroup frequencies were compared with those in modern Eurasian populations, including the populations of Volga-Ural region by Principal Component Analysis (PCA) performed using Excel implemented with XlStat.

Results and Discussion

From the analysis of 228 clone sequences we have obtained the mtDNA HVS-I in 13 individuals, showing 12 independent haplotypes. They were further classified into 10 mtDNA haplogroups: H, H1, H5, H13, HV1, J, J1, T, T2 and U3 (Table 2; Achilli et al. 2007; Karachanak et al. 2012; Richards et al. 2002; Soares et al. 2010; Torroni et al. 2001).

We compared haplogroups in our ancient samples to those in modern Bulgarian samples previously analyzed (Karachanak et al., 2012).

The main haplogroup H, prevalent in European populations has 41.9 % frequency in modern Bulgarians (Karachanak, S. et al., 2012) and it is observed in seven out of thirteen proto-Bulgarian samples.

The rest of the ancient mtDNAs belong to one of the following Western Eurasian haplogroups HV1, J, J1, T, T2 and U3. They are found in modern Bulgarians with frequencies of: 0.2%, 7.9%, 1.3%, 10.6%, 6.3% and 1.9%, respectively. We found no evidence of East Asian (F, B, P, A, S, O, Y or M derivative) and African (L) haplogroups. Thus, our results do not support theories of Mongolo-Altaic and Hun-Tataric origins of proto-Bulgarians.

The PCA analysis of modern Eurasian populations, including Volga-Ural populations and Proto-Bulgarians is based on mtDNA haplogroup frequencies given in Supplementary Table. The PCA plot (Fig. 2) shows that from mtDNA perspective the Proto-Bulgarians are positioned among South-Eastern and Southern European populations including modern Bulgarians. Proto-Bulgarians are genetically distant from Northern and Western Europeans and populations from the Near East and Caucasus. On the greatest distance from Proto-Bulgarians are Volga-Ural and Arabic populations.

Our results therefore suggest that proto-Bulgarians are genetically similar to modern Bulgarians and to certain South-Eastern European as well as Italian populations.

The future analyses of samples from human remains found on the territory of Bulgaria and dating to different periods (since III millennium BC) will further clarify the genetic make-up of past populations inhabiting modern Bulgarian lands.

Conclusion

The range of the molecular anthropological research has increased in recent years due to the extensive research of the origins of modern and past populations. The results create a map of possible prehistoric human migration routes at different time scales and provide a detailed reconstruction of prehistoric and historic events all over the world. Thus, ancient and modern

data create a picture of our history since the appearance of modern humans 200,000 years ago in East Africa.

This work on ancient Bulgarian samples adds to the genetic picture of the past by presenting the first data on ancient mtDNA samples from individuals who inhabited the current Bulgarian territories from VIII-X century AD. Our results show that the haplogroups found in ancient samples are predominantly Western Eurasians. This finding supports the concept for the Western Eurasian matrilineal origins of the Proto-Bulgarians and is controversial to the Mongolo-Altaic and Hun-Tataric theories. The comparison of Proto-Bulgarians and modern Eurasian populations, including those form the Volga-Ural region shows that despite the time gap of more than eleven centuries, there is a genetic similarity between proto- and modern Bulgarians (Karachanak et al. 2012).

Phylogenetic analysis of additional human remains will help to further clarify the gradual changes in the matrilineal composition of past populations inhabiting modern Bulgarian lands. This data will contribute to a deeper understanding of the Bulgarian genetic past.

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Table 1. Description of Samples from Three Necropolises in Bulgaria

Sample	Necropolis	Grave	Location/Description	Dating
NJ 50	Nojarevo	No. 50	Northeast Bulgaria Nojarevo Village/ Proto-Bulgarian Separate graves	VIII-IX Century
NJ 54	Nojarevo	No. 54		
NJ 84	Nojarevo	No. 84		
NJ 77	Nojarevo	No. 77		
NJ 125	Nojarevo	No. 125		
TUH 448	Tuhovishte	No. 448	Southwest Bulgaria Tuhovishte Village/ Proto-Bulgarian Separate graves	IX-X Century
TUH 449	Tuhovishte	No. 449		
TUH 1649	Tuhovishte	No. 1649		
TUH 1652	Tuhovishte	No. 1652		
TUH 1665	Tuhovishte	No. 1665		
MM 1.2	Monastery of Mostich	No. 5	Northeast Bulgaria Veliki Preslav/ Proto-Bulgarian Separate graves	X Century
MM 1.3	Monastery of Mostich	No. 25		
MM 1.4	Monastery of Mostich	No. 5		

Table 2. Haplotypes and Haplogroups in Proto-Bulgarians

Sample	Clones	HVS-I haplotypes	Sequence range	Hg	Sub-Hg	HaploGrep quality score
MM 1.2	19	092 142 266 278	16024-16383	H1	H1t1a1	60
MM 1.3	15	343	16225-16383	U3	U3	100
MM 1.4	19	069 126	16024-16281	J	J	100
NJ 50	18	111 173 183 278	16024-16383	H1	H1r1	61
NJ 54	18	069 126 145 172 222 261	16024-16383	J1	J1b1a1	100
NJ 77	10	055 067	16024-16156; 16225-16383	HV1	HV1	100
NJ 84	22	126 274 294 296	16024-16383	T2	T2	91
NJ 125	14	CRS	16024-16281	H	H2a2a1	0
TUH 448	11	148 256	16024-16281	H13	H13a2c1	100
TUH 449	22	304	16024-16383	H5	H5	100
TUH 1649	19	CRS	16024-16383	H	H2a2a1	0
TUH 1652	18	126 294	16024-16383	T	T	100
TUH 1665	23	219 325	16024-16383	H1	H1a2	69

MM = Monastery of Mostich necropolis; NJ = Nojarevo necropolis; TUH = Tuhovishte necropolis; Hg = haplogroup; CRS = Cambridge reference sequence

Figure 1. Physical map of Bulgaria showing the location of the three necropolises from which the human remains were collected (Nojarevo dated to VIII-IX century, Tuhovishte-IX-X and Monastery of Mostich – X century).

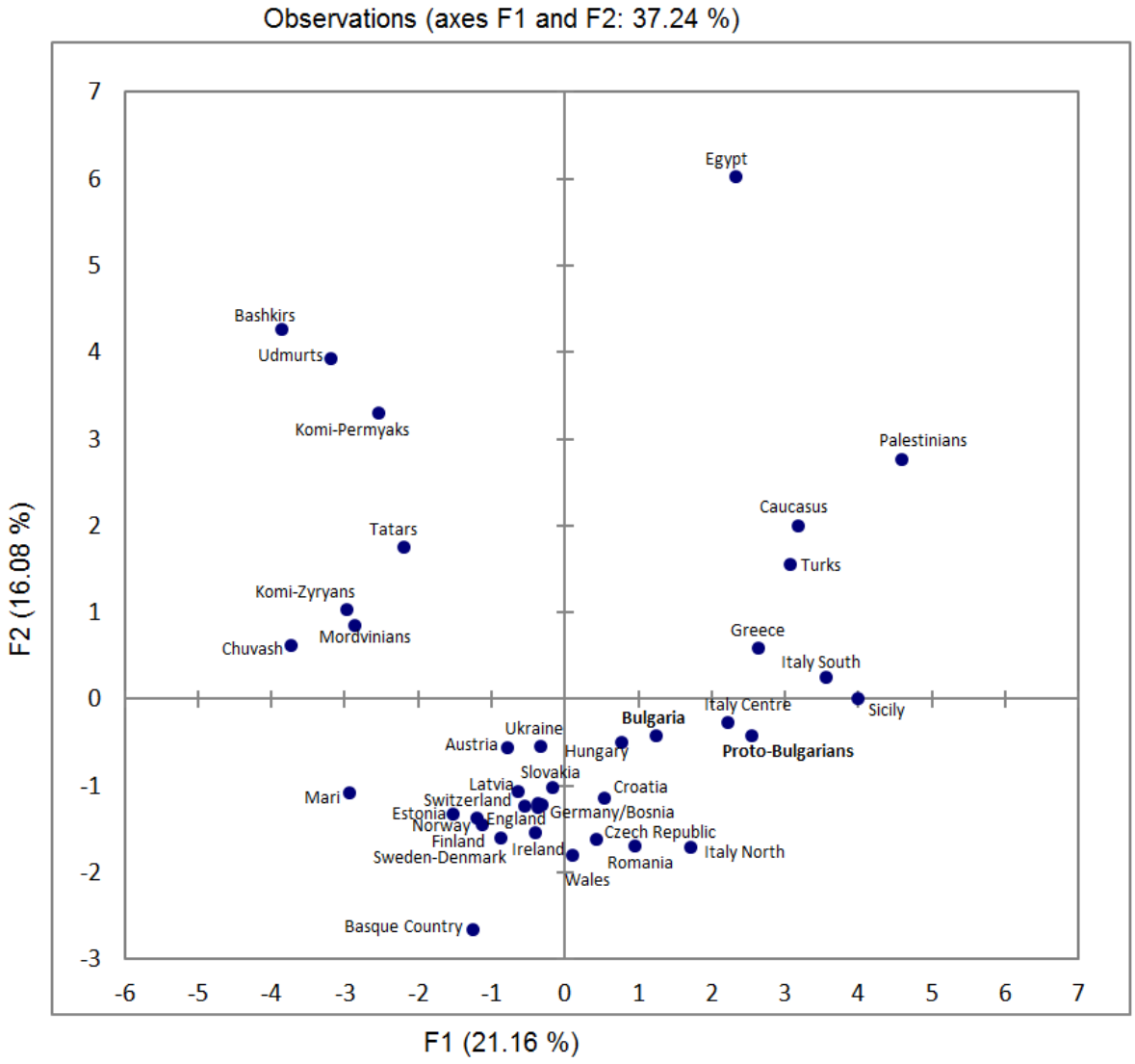

Figure 2. Plot of the Principal Component Analysis based on mtDNA haplogroup frequencies in Eurasian populations.

Supplementary Table. Populations included in the PCA, number of samples analyzed (N) and number of individuals belonging to mtDNA haplogroups.

Popul.	References	N	H*	H5	HV0	HV	R0a	JT	U1	U2e	U3	U4	U5a	U5b	U6	U7	U8	U*	K	N1	N2	X	M	L	Others
Austria	Karachanak et al., 2012	99	43	3	1	1	1	20*	0	1	1	4	8	0	0	0	2	1	7	2	1	1	2	0	0
Basque Country	Karachanak et al., 2012	156	87	6	17	0	0	13*	0	0	0	0	2	17	0	1	1	0	6	0	0	2	0	1	3
Bosnia	Karachanak et al., 2012	144	61	8	9	0	2	17*	2	0	1	8	10	7	0	0	0	0	6	5	2	2	2	1	1
Bulgaria	Karachanak et al., 2012	996	380	33	35	39	6	182*	13	10	21	39	45	25	0	7	4	3	59	27	25	20	11	4	8
Caucasus	Karachanak et al., 2012	2650	573	61	24	104	7	439*	108	60	147	104	153	27	1	25	13	1	159	56	93	145	163	2	185
Croatia	Karachanak et al., 2012	96	36	7	5	3	0	9*	1	4	2	2	8	2	0	1	0	0	6	3	4	0	2	0	1
Czech Repu	Karachanak et al.,	83	28	6	5	3	0	18*	0	1	1	1	7	3	0	0	0	0	3	3	1	3	0	0	0

blic	2012																							
Egypt	Karachanak et al., 2012	413	15	2	13	14	7	55*	2	1	14	5	1	15	3	2	0	0	23	27	4	7	41	94	68
England	Karachanak et al., 2012	335	148	13	11	1	0	75*	0	3	2	7	13	15	0	1	1	0	21	10	3	3	0	2	6
Estonia	Karachanak et al., 2012	558	235	17	18	6	0	98*	1	7	5	32	56	24	0	0	9	1	15	13	15	5	1	0	0
Finland	Karachanak et al., 2012	312	113	8	19	0	0	37*	1	2	0	5	18	44	0	1	0	0	19	17	16	6	0	1	5
Germany	Karachanak et al., 2012	905	368	43	37	3	0	177*	4	2	16	26	46	35	0	2	2	3	63	24	21	10	2	1	20
Greece	Karachanak et al., 2012	155	54	6	3	5	3	31*	3	1	3	4	7	4	0	2	0	1	7	7	2	7	4	0	1
Hungary	Karachanak et al., 2012	533	136	21	25	4	7	75*	4	4	1	9	15	17	1	1	11	2	113	28	28	7	11	2	11
Ireland	Karachanak et al., 2012	300	126	5	17	4	1	54*	0	4	3	4	11	15	0	0	0	0	37	9	7	2	1	0	0

Italy Centre	Karachanak et al., 2012	1273	427	52	61	45	14	251*	11	10	34	21	56	34	4	14	10	1	84	35	25	39	25	18	2
Italy North	Karachanak et al., 2012	346	131	33	18	8	1	53*	3	5	4	10	7	4	0	0	1	0	32	12	6	17	1	0	0
Italy South	Karachanak et al., 2012	539	209	25	19	18	5	93*	10	4	17	13	11	7	7	6	2	0	39	21	9	14	7	1	2
Latvia	Karachanak et al., 2012	299	113	20	9	7	0	47*	0	9	5	28	21	6	0	0	0	0	7	13	12	1	1	0	0
Norway	Karachanak et al., 2012	556	250	17	21	1	0	106*	1	0	8	17	35	31	0	0	2	0	31	13	9	2	6	0	6
Palestinians	Karachanak et al., 2012	117	28	5	0	2	3	26*	1	1	1	2	1	0	1	3	0	1	9	3	3	4	4	16	3
Romania	Karachanak et al., 2012	94	26	7	6	1	2	21*	0	0	3	4	7	5	0	0	0	0	3	0	6	2	0	0	1
Sicily	Karachanak et al., 2012	105	51	6	3	4	2	13*	2	1	1	3	1	1	1	2	0	0	6	3	0	3	0	2	0
Slova	Karach	12	49	9	3	2	0	30*	0	2	3	2	9	2	0	0	0	0	5	6	3	0	1	0	3

kia	anak et al., 2012	9																						
Sweden-Denmark	Karachanak et al., 2012	75	31	4	3	0	0	16*	0	1	0	3	5	0	0	0	0	0	8	1	1	0	0	0	2
Switzerland	Karachanak et al., 2012	228	93	11	11	1	0	54*	0	2	2	8	11	5	1	0	1	0	12	3	4	1	0	1	7
Turks	Karachanak et al., 2012	340	99	10	2	17	0	61*	11	4	19	5	4	6	0	2	4	2	19	13	10	15	15	7	15
Wales	Karachanak et al., 2012	92	43	7	3	2	0	18*	0	0	0	0	3	3	0	0	0	0	7	3	0	1	0	0	2
Bashkirs	Bermisheva et al. 2002	221	25	2	7	1	0	19	0	0	0	28	15	15	0	0	1	0	3	11	1	0	61	0	33
Tatars	Bermisheva et al. 2002	228	68	2	9	1	0	38	2	0	5	16	20	4	0	0	0	5	13	7	4	0	20	0	17
Chuvash	Bermisheva et al. 2002	55	15	0	4	0	0	5	0	0	1	9	8	0	0	0	1	1	4	2	1	0	4	0	0
Mordvinia	Bermisheva et	102	42	1	5	1	0	16	0	1	0	2	7	9	0	0	0	2	0	6	0	0	3	0	7

ns	al. 2002																							
Komi-Permyaks	Bermisheva et al. 2002	74	22	2	0	0	0	13	0	0	0	7	1	3	0	0	0	1	1	9	0	0	12	0	6
Komi-Zyryans	Bermisheva et al. 2002	62	21	0	0	0	0	14	0	0	0	15	2	4	0	0	1	0	1	0	1	0	2	0	5
Mari	Bermisheva et al. 2002	136	54	1	15	2	0	17	0	0	0	14	17	2	0	0	0	0	3	1	0	0	8	0	2
Udmurts	Bermisheva et al. 2002	101	21	1	0	0	0	23	0	5	0	4	8	1	0	0	0	2	0	0	0	0	20	0	11
Ukraine	unpiblished	110	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub	unpub.
Proto-Bulgarians	present study	13	6	1	0	1	0	4	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1

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*Haplogroup JT includes haplogroup J, T1 and T2