

Mitochondrial DNA Phylogeny in Eastern and Western Slavs

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To resolve the phylogeny of certain mitochondrial DNA (mtDNA) haplogroups in eastern Europe and estimate their evolutionary age, a total of 73 samples representing mitochondrial haplogroups U4, HV*, and R1 were selected for complete mitochondrial genome sequencing from a collection of about 2,000 control region sequences sampled in eastern (Russians, Belorussians, and Ukrainians) and western (Poles, Czechs, and Slovaks) Slavs. On the basis of whole-genome resolution, we fully characterized a number of haplogroups (HV3, HV4, U4a1, U4a2, U4a3, U4b, U4c, U4d, and R1a) that were previously described only partially. Our findings demonstrate that haplogroups HV3, HV4, and U4a1 could be traced back to the pre-Neolithic times (~12,000–19,000 years before present [YBP]) in eastern Europe. In addition, an ancient connection between the Caucasus/Europe and India has been revealed by analysis of haplogroup R1 diversity, with a split between the Indian and Caucasus/European R1a lineages occurring about 16,500 years ago. Meanwhile, some mtDNA subgroups detected in Slavs (such as U4a2a, U4a2*, HV3a, and R1a1) are definitely younger being dated between 6,400 and 8,200 YBP. However, robust age estimations appear to be problematic due to the high ratios of nonsynonymous to synonymous substitutions found in young mtDNA subclusters.

Introduction

According to archaeological data, the eastern part of Europe was colonized by modern humans about 42,000–45,000 years before present (YBP), probably as early as anywhere else in northern Eurasia (Anikovich et al. 2007). Genetic data suggest that maternal mitochondrial DNA (mtDNA) lineages can be traced farther back into prehistory, through the Last Glacial Maximum (LGM), to the first settlement of Europe by anatomically modern humans almost 50,000 YBP (Richards et al. 2000). The LGM took place between 19,000 and 22,000 YBP, and during this interval European population was most probably concentrated in refugia in the western Caucasus and southern European peninsulas (Peyron et al. 1998; Tarasov et al. 2000). Thus, a model of postglacial expansion–recolonization from refugia appears to be a major concept to explain the genetic diversity of the present-day Europeans (Torrioni et al. 1998; Rootsi et al. 2004; Roostalu et al. 2007).

A founder analysis of mtDNA lineages in populations of Near East and Europe has demonstrated that the majority of extant mtDNA lineages entered Europe in several episodes during the Upper Palaeolithic (Richards et al. 2000). Neolithic founder clusters (9,000 YBP) were mostly members of haplogroups J, T1, and U3. The main contributors to the late Upper Paleolithic expansions (14,500 YBP) were the major subclusters of haplogroups H, K, T*, T2, W, and X. The main components of the middle Upper Paleolithic (26,000 YBP) were HV*, U1, possibly U2, and U4, and the main component of the early Upper Paleolithic (45,000 YBP) was mainly haplogroup U5 (Richards et al. 2000). The age estimates

for the major mtDNA clusters in Europe and Near East are consistent with assumption that these clusters are considerably older in the Near East, but there is also the possibility that significant dispersals may have originated in northern Caucasus and eastern Europe as has been suggested for the middle Upper Paleolithic component (Soffer 1987). It is known that mtDNA haplogroup U4 is present in Finno-Ugric- and Turkic-speaking populations of eastern Europe at a frequency of about 9%, whereas HV* is infrequent, about 0.5% (Bermisheva et al. 2002). In Russian populations, the picture is somewhat different—haplogroups U4 and HV* are present, on average, at frequencies of 3.5% and 2.5%, respectively (Malyarchuk, Grzybowski, et al. 2002; Malyarchuk et al. 2004). These values are comparable with those described for other European populations (Richard et al. 2007).

High mutation rates and homoplasy in the mtDNA control region appear to be an obstacle to developing a reliable classification of mitochondrial lineages and in identifying the correct dating of mtDNA clades (Bandelt et al. 2002; Malyarchuk, Rogozin, et al. 2002). Hence, complete mtDNA sequences may represent the best possible solution for phylogeographic analysis (Torrioni et al. 2006). However, until now, the reconstruction of the phylogeny of haplogroups U4 and HV* has been only partially performed at the level of complete genome resolution (Achilli et al. 2004, 2005; Palanichamy et al. 2004). Therefore, the objective of this study is to provide new information concerning the molecular dissection of haplogroups U4 and HV* present in eastern European populations, such as Russians, Belorussians, Ukrainians, Poles, Czechs, and Slovaks. Moreover, we present here the results of the complete genome characterization of the R1 haplogroup (one rarely seen in Europe) encouraged by its occurrence in the northwestern Russian populations (Malyarchuk et al. 2004; Grzybowski et al. 2007). The vast majority of the complete genomes displayed here belong to novel subhaplogroups, informative in terms of updating the west Eurasian phylogeny and the understanding of some aspects of the genetic history of Europeans.

Key words: human mitochondrial DNA, complete genome sequencing, population genetics, molecular phylogeography, eastern Europe, Slavonic populations.

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Table 1
Haplogroups U4a1 and U4a2 Distribution (Percentage with Number of Individuals in Parentheses) in Different Populations of Eastern, Central, and Western Europe

Populations	U4a1	U4a2	U4a2a	U4a2b	U4a2c
Eastern Europe ($n = 4,008$) ^{a,b,c}	1.8 (74)	1.4 (57)	0.8 (31)	0.3 (9)	0.3 (13)
Central and eastern Europe ($n = 2,016$) ^a	1.0 (21)	1.9 (38)	1.2 (24)	0.4 (7)	0.1 (3)
Northeastern Europe ($n = 1,013$) ^b	0.9 (9)	0.8 (8)	0.6 (6)	0.2 (2)	0
Volga–Ural region ($n = 979$) ^c	4.5 (44)	1.1 (11)	0.1 (1)	0	1.0 (10)
Central and western Europe ($n = 3,704$) ^d	0.5 (19)	0.3 (12)	0.2 (9)	0.1 (2)	0

NOTE.—The data were taken from the literature cited in supplementary table S2 (Supplementary Material online).

^a Russians, Belorussians, Ukrainians, Poles, and Slovaks.

^b Finns, Karelians, Estonians, Latvians, and Lithuanians.

^c Maris, Komi-Zyrians, Komi-Permians, Mordwin, Udmurts, Chuwashees, Tatars, and Bashkirs.

^d British, Germans, Czechs, Austrians, Swiss, Bosnians, Slovenians, Italians, French, Spaniards, and Portuguese.

by the control region transition at np 310, and U4a3 that has been found only once in our study in Czech population and, therefore, cannot be defined for the time present by minimal nucleotide motif.

Haplogroup U4a1 is largely characterized by an eastern European and a west Siberian distribution being found at highest frequencies (7–21%) in populations such as Mari, Chuvash, and Kets (table 1, supplementary table S2, Supplementary Material online). We investigated the structure of haplogroup U4a1 by complete genome sequencing of 12 mtDNAs from populations of Poles, Czechs, Slovaks, Belorussians, and Russians. The resulting tree (fig. 1) indicates that there are at least 3 subclusters within haplogroup U4a1–U4a1a, with transition at np 961 and insertion of 3 C's at np 965, and its subclade U4a1a1 defined by additional transitions at nps 8167 and 12618 and insertion of 1 C at np 5899; U4a2 defined by transitions at nps 745 and 3204; and U4a1c characterized by a rank of mutations at nps 8155, 13158, 14110, and 16234. Interestingly, among eastern Slavs (Russians and Belorussians) the only specific subclade U4a1a1 has been revealed, whereas in western Slavs the remaining subclades have been found. The coalescence time estimate for U4a1 complete genomes was $14,650 \pm 2,400$ YBP. This value is close to those (around 10,000–14,000 YBP) calculated from the first hypervariable segment (HVS1) data for central European (Germanic-speaking) populations but not for the Baltic Finno-Ugric and Volga people (around 20,000–22,000 YBP) (Tambets et al. 2003).

Complete genome sequencing of 26 mtDNAs has shown that haplogroup U4a2 comprises at least 3 subclades—U4a2a, with a back mutation at np 16356; U4a2b, characterized by transition at np 16223; and U4a2c, with diagnostic coding region transitions at np 8567. Its major subcluster U4a2c1 (marked by the variants 10654, 16242A, 16288, and 16362) was observed at elevated frequencies (0.9–3.6%) in Turkic-speaking groups of the Volga basin (Bermisheva et al. 2002), whereas earlier branch of U4a2c was detected in our study in the Belorussian population (SV8 in supplementary table S1, Supplementary Material online; fig. 1). In addition, a number of unclassified U4a2* haplotypes have been revealed, mainly among Slovaks and Russians (fig. 1).

Based on the combined presence of transitions at nps 7705 and 11339, Achilli et al. (2005) identified haplogroup

U4b on the basis of complete mtDNA of an Adygei individual. In our samples from eastern Europe, we completely sequenced 4 samples with these polymorphisms. Interestingly, one of these samples was lacking the 11339 transition. Therefore, we propose to redefine haplogroup U4b by the 7705 transition and nominate lineages inside U4b with the transition at np 11339 as U4b1.

In our study, we identified a novel haplogroup U4c, which can be recognized by the 10907 transition. This transition is among root markers of the clade (at positions 4811, 6146, 9070, 10907, and 14866) represented in the MitoMap mtDNA tree (Ruiz-Pesini et al. 2007) by several coding region sequences reported previously by Herrnstadt et al. (2002) and Kivisild et al. (2006). For this reason, we propose to name U4c subclade defined by mutations at positions 4811, 6146, 9070, and 14866 as U4c1. Another new haplogroup is U4d, which is recognizable by 2 mutations—a transition at np 629 and insertion of 1 C at np 2405. However, the contemporary geographic distribution of this haplogroup is uncertain due to lack of the control region markers.

Using the calibration method of Mishmar et al. (2003), the coalescence age for haplogroup U4 is $20,460 \pm 1,300$ YBP, which is consistent with the age of U4 (around 16,000–24,000 YBP) estimated previously by Richards et al. (2000) on the basis of HVS1 sequence variation. Coalescence time estimates for haplogroups U4a and U4a2 were $12,100 \pm 1,200$ and $7,300 \pm 1,180$ YBP, respectively. Within U4a2, subclades U4a2a (12 genomes) and U4a2* (8 genomes) coalesce at $6,425 \pm 1,640$ and $7,068 \pm 2,134$ YBP, respectively. However, one should note that there are considerable differences between U4 clades in the non-synonymous versus synonymous mutations ratio (table 2, supplementary table S3, Supplementary Material online). This ratio as estimated on the tree (fig. 1) varies from 0.2–0.5 in haplogroups U4d, U4b, U4c, and U4a1 to 1.1 in U4a2. Previous studies have shown that the number of nonsynonymous substitutions increases from the average of 0.4 in “older” mtDNA clades to 0.62 in “younger” ones (Kivisild et al. 2006). Hence, a significant excess of nonsynonymous mutations in U4a2 can be explained by its young age. Using a mutation rate counting only synonymous substitutions (Kivisild et al. 2006), the age for U4a2 is only $3,100 \pm 880$ YBP, which is broadly consistent with the beginning of Slavonic prehistory, according to data

Table 2
Diversity and the Rate of Nonsynonymous/Synonymous Changes in Haplogroups U4, HV3, HV4, and R1

Haplogroup	<i>N</i>	Diversity ($\rho \pm$ s.e.) ^a	<i>M_N</i>	<i>M_S</i>	<i>M_N/M_S</i>	Diversity ($\rho \pm$ s.e.) ^b
U4	49	3.98 ± 0.26	26	52	0.5	
U4a	42	2.36 ± 0.24	17	25	0.68	0.78 ± 0.14
U4a1	13	2.85 ± 0.47	5	10	0.5	
U4a2	26	1.42 ± 0.23	12	11	1.09	0.46 ± 0.13
U4a2a	12	1.25 ± 0.32	4	5	0.8	0.42 ± 0.19
U4a2*	8	1.38 ± 0.4	2	4	0.5	
U4b	4	4.0 ± 1.0	3	10	0.3	
U4c	4	5.75 ± 1.2	4	8	0.5	
U4d	6	5.0 ± 0.91	2	9	0.22	
HV3	17	2.47 ± 0.38	7	17	0.41	
HV3a	5	1.6 ± 0.57	1	6	0.17	
HV3b	4	3.0 ± 0.87	3	3	1.0	1.75 ± 0.66
HV4	4	3.75 ± 0.97	5	6	0.83	1.75 ± 0.66
R1	6	5.5 ± 0.96	5	8	0.63	3.83 ± 0.8
R1a	5	3.2 ± 0.8	3	5	0.6	1.6 ± 0.57
R1a1	4	1.25 ± 0.6	1	2	0.5	

NOTE.—s.e., standard deviation of the ρ estimate.

^a Diversity has been calculated taking into account all coding region substitutions.

^b Diversity has been calculated taking into account only synonymous substitutions due to the high (>0.6) values of the M_N/M_S proportion.

of archeology and linguistics (Gimbutas 1971; Sedov 1979).

Haplogroups HV3 and HV4 Phylogeny

The majority of west Eurasian mtDNA haplogroups consist of a small number of phylogenetically well-characterized branches of haplogroup R, such as JT, U, and HV. Among them, haplogroup HV defined by substitutions at nps 73, 11719, and 14766 in relation to R* root is represented by haplogroups, H, HV0 (including V), and several less studied branches, namely HV1, HV2, HV3, and still unclassified HV*. It has been suggested that most of the HV haplogroups presently found in Europe originated in the Near East and Caucasus region (Richards et al. 2000; Tambets et al. 2000), but there are still many questions concerning classification of haplogroups belonging to HV family.

In the eastern European populations studied, haplogroup HV1 defined by HVS1 region transition at np 16067 has been revealed at low frequency (0.2%) only in Poles (Malyarchuk, Grzybowski, et al. 2002). It is known that in Europe, low frequencies of this haplogroup can be seen in populations of Romania, Bulgaria, and Hungary (Richards et al. 2000; Egyed et al. 2007). Haplogroup HV2 defined by HVS1 region mutation at np 16217 is also very rare in European populations being found in single instances in populations such as Slovaks and Bosnians (Malyarchuk et al. 2003, 2008), but in the Indo-Pakistani region its frequency is very high (about 10%) (Metspalu et al. 2004; Quintana-Murci et al. 2004). In a large Russian population, we have not detected any HV1 and HV2 sequences but have found many haplotypes defined by the HVS1 region mutation at np 16311. These haplotypes are assigned to the haplogroup HV3 as suggested by Metspalu et al. (2004), although such grouping may be artificial due to mutational instability of np 16311 (Bandelt et al. 2002; Malyarchuk, Rogozin, et al. 2002). HV3 haplotypes have been observed with low frequencies (<1%) in populations of north central and northwestern Europe,

whereas its contribution to the mtDNA pools of Mediterranean and southeastern Europeans appears to be slightly higher (1–1.3%) (Richards et al. 2000). The highest frequencies of HV3 (2.3–5.8%) have been detected thus far in some populations of the Near East, Anatolia, Iran, and Iraq (Richards et al. 2000; Al-Zahery et al. 2003; Metspalu et al. 2004; Quintana-Murci et al. 2004). In northeastern Europe, the highest frequency of HV3 (2.9%) has been observed in Latvians, although it is represented there only by the nodal HVS1 haplotypes (Pliss et al. 2006). Similarly, individual nodal HVS1 haplotypes belonging to the HV3 have been encountered in several populations of the Volga basin (Bermisheva et al. 2002). Among the Slavs, haplogroup HV3 contributes equally to the Russian, Czech, and Slovak mtDNA pools (in average, 1.8%) but essentially less (0.5%) to the gene pool of Poles (these differences are statistically significant only for Russian–Polish comparison, $P = 0.015$).

Complete mitochondrial genome data from eastern European populations allow refinement of the haplogroup HV phylogeny. As shown in figure 2, except for HV3, other haplogroups can be identified—HV4, characterized by the coding region transition at np 7094, and its subcluster HV4a, which can be recognized by the coding region transition at np 709. A novel monophyletic subclade defined by transition at np 8994 emerged within HV3 and is called HV3a here. The mitochondrial genome #As37 has been assigned to HV3a only conditionally (based on transition at np 8994) because only the coding region information is available for this sample and the presence of transition at np 16311 is not verified (Kivisild et al. 2006). In addition, another new clade inside haplogroup HV3, HV3b, is recognizable by 2 transitions at nps 6755 and 16172. A major subcluster within HV3b is HV3b1 defined by transversion from A to C at np 16113 and 3 transitions at nps 3507, 8545, and 9266. We have found also that Russian HVS1 sequences with motif 16278–16311 are clustered in subgroup HV3c by transitions at nps 5471 and 14560. One more subcluster, HV3d joins 2 samples (Russian and Slovak ones)

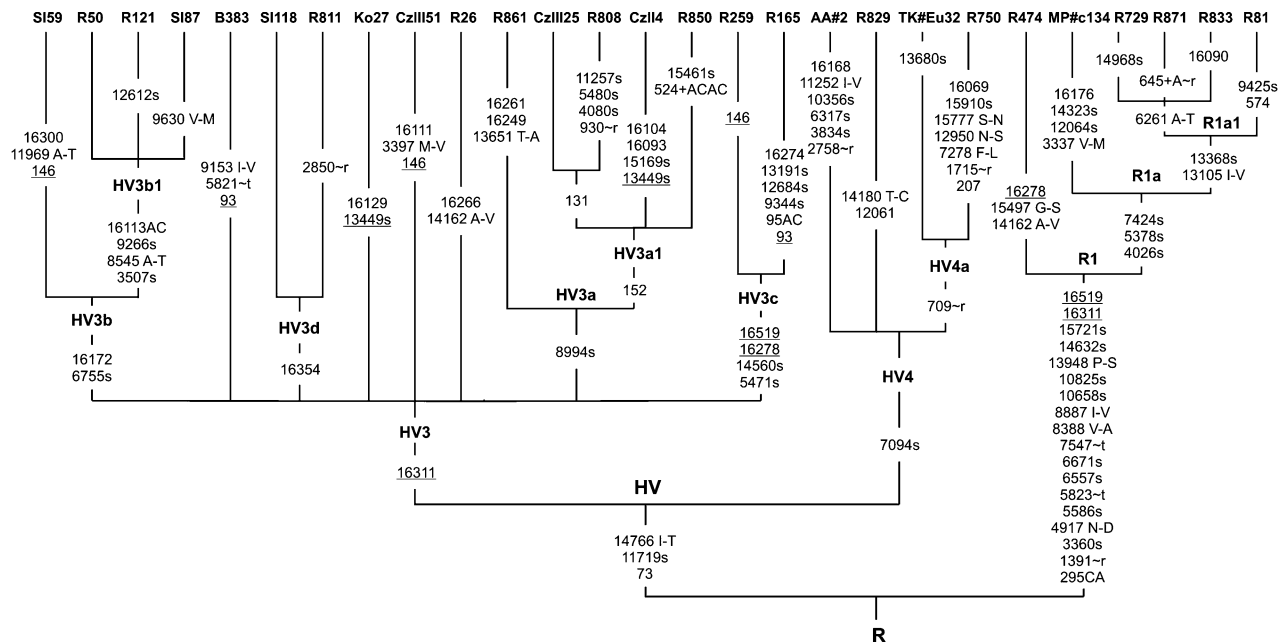


FIG. 2.—Most parsimonious tree of the complete haplogroups HV3, HV4, and R1 sequences rooted in haplogroup R. The tree includes mtDNAs designated in accordance with population codes explained in the legend of figure 1. Sequences taken from the literature are designated as follows: TK (only coding region information is available), Kivisild et al. (2006); MP, Palanichamy et al. (2004).

by the 16354 transition in HV3S1. The remaining completely sequenced HV3 lineages simply branch off from the HV3 root.

The coalescence age of the entire HV3 defined by a transition at np 16311 is $12,700 \pm 1,960$ YBP. We should note, however, that HV3 might be polyphyletic due to the hypervariability of np 16311. Meanwhile, subclusters HV3a and HV3b are likely monophyletic and their age estimates were $8,200 \pm 2,900$ and $15,420 \pm 4,500$ YBP, respectively. However, HV3b is characterized by a high ratio of nonsynonymous versus synonymous substitutions, so its coalescence age can be estimated as $11,837 \pm 4,460$ YBP, using the rate suggested by Kivisild et al. (2006) (table 2). The same is probably true for the haplogroup HV4 age estimation.

Refinement of the Haplogroup R1 Phylogeny

There is very little complete mitochondrial sequence data concerning haplogroup R1, very rarely observed in European populations (Richards et al. 2000). In eastern Europe, R1 haplotypes were encountered only in northwestern Russians and Poles (Malyarchuk et al. 2004; Grzybowski et al. 2007). To date, only one complete mtDNA sequence belonging to this clade has been published from the Brahmin population of India (sample C134; Palanichamy et al. 2004). Five additional genomes presented here allow us to refine the R1 phylogeny (fig. 2). The root of R1 can now be defined by 15 coding region mutations, whereas 3 transitions at nps 4026, 5378, and 7424 separate subcluster R1a from haplotypes characterized by transitions at nps 14162, 15497, and 16278. In turn, subcluster R1a consists of 2 clades—an Indian one represented by sample C134 (Palanichamy et al. 2004) and the Caucasus/European

one, R1a1, which is defined by the 13105 and 13368 transitions. It should be noted that haplogroup R1 was described for the first time in Adygei from the northern Caucasus (Macaulay et al. 1999). Its presence, albeit at low frequencies, was then confirmed in some populations of the Caucasus (e.g., in the Kabardins which are linguistically related to the Adygei people) (Nasidze and Stoneking 2001), the Near East (Richards et al. 2000; Rowold et al. 2007), and the south Caspian region (in Iran and Turkmenistan) (Metspalu et al. 2004; Quintana-Murci et al. 2004).

It has been suggested that haplogroup R1 and other haplogroups rarely observed in European populations (R2, N1a) were brought to Europe from the Near East in the Neolithic times (Torroni et al. 2006). Meanwhile, the complete sequencing of R1 mtDNAs suggests a deep split between the more ancient 16278–16311 R1 branch and the R1a subcluster, about $28,300 \pm 4,900$ YBP (fig. 2). A second split (about $16,450 \pm 4,100$ YBP) is seen between the Indian haplotype C134 and the R1a1 subcluster. Both R1 types are present in Adygei population of the northern Caucasus (Macaulay et al. 1999), thus suggesting that R1 evolution occurred in the Caucasus area, from where these lineages have extended in different directions. It is known that the Adygei people (the Adygei, Cherkess, and Kabardins) are one of the most ancient indigenous populations of the Caucasus region. Therefore, the presence of R1 haplotypes in populations of northwestern Russians can be explained by the contribution of northern Caucasus populations to the Russian gene pool.

Discussion

Although the previous studies of mtDNA diversity in European populations suggested that both haplogroup

frequency patterns and haplotype composition in Slavs were similar to those characteristic of other Europeans, complete genome sequencing allowed us to describe the specific mtDNA components which are found predominantly in modern-day Slavs, albeit introduced at different times into European gene pools. The results of this study show that some of them (HV3, HV4, R1, and U4a1) could possibly be traced back to the pre-Neolithic in eastern Europe and the Caucasus area. These mtDNA lineages were probably involved in the late-glacial expansions from eastern European refugia after the LGM. The estimated split between the Indian and Caucasus/European R1a branches dated as ~16,500 YBP confirms this idea, but more data on the diversity of haplogroup R1 in different Eurasian populations are needed.

Meanwhile, some of mtDNA subgroups widespread in Slavonic populations (such as U4a2a, U4a2*, HV3a, and R1a1) are definitely younger (dated between 6,400 and 8,200 YBP), suggesting that their expansions may be related to more recent historical events. Yet the most important component from the viewpoint of ethnic history of Slavs is mitochondrial subcluster U4a2, most probably of a central eastern European origin. Expansion of this subcluster may be explained by a dispersal of the Corded Ware culture, which flourished 5,200–4,300 YBP in eastern and central Europe. This culture (also known as the Battle-Axe culture) encompassed most of continental northern Europe from the Volga River in the east to the Rhine River on the west (Gimbutas 1971; Mallory 1989).

However, the results of genetic dating should be interpreted with caution because of the problems with the rate of mutations and possible influence of selection on the evolution of mtDNA (Torroni et al. 2001; Mishmar et al. 2003). Haplogroups U4a2, HV3b, HV4, and R1 demonstrate a substantial fraction of nonsynonymous mutations whose proportion to synonymous ones varies from 0.63 to 1.1. For instance, $Ka/(Ka + Ks)$ ratio for 25 U4a2 genomes showing the highest M_N/M_S ratio was 0.31, whereas it was only 0.17 for all 49 U4 genomes studied. Meanwhile, within haplogroup U4 protein-coding genes the pattern of nonsynonymous and synonymous changes was different because $Ka/(Ka + Ks)$ ratios varied from 0.46 and 0.27 in *ND5* and *ATP6* to 0.1 and 0.03 in *ND4* and *COI*, respectively. It is well-known that the *ATP6* gene has the highest level of nonsynonymous substitutions in global mtDNA comparisons (Mishmar et al. 2003; Elson et al. 2004), but *ND5* is usually less variable and even conservative, as in haplogroup J (Moilanen and Majamaa 2003). In this regard, we hope that further extending of the haplogroup U4 complete genome data sets will help to solve the question of whether or not adaptive selection acts on the *ND5* gene in European haplogroup U4.

Due to the relatively high rates of nonsynonymous to synonymous substitutions detected in some mtDNA subclusters, the mutation rate counting only synonymous substitutions can be used in such cases for the time estimates (Kivisild et al. 2006). This approach allowed lower estimations of the coalescence times for certain mtDNA clusters (table 2), pointing even to the possibility that the flowering of some mtDNA clades (such as U4a2 and U4a2a) might be due to the recent expansion (~3,000 YBP) of pre-Slavonic

tribes in central and eastern Europe. Therefore, it seems crucial for the future to develop general principles for using an mtDNA coding region molecular clock in dating events in human population history.

Supplementary Material

Supplementary tables S1, S2, and S3 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>). All completely sequenced mitochondrial genomes have been submitted to the GenBank database, under accession numbers EF222232–EF222253 and EU545415–EU545465.

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