

1 **Evidence for Early European Neolithic Dog Dispersal: New Data on South-Eastern**

2 **European subfossil dogs from Prehistory and Antiquity Ages**

3 **Short title: South-Eastern European subfossil dogs**

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Abstract

Objectives

The history of dog domestication is still under debate, but doubtlessly, it is a process of an ancient partnership between dogs (*Canis familiaris*) and humans. Although data on ancient DNA dog diversity are scarce, it is clear that several regional dog populations had been formed in Eurasia up to the Holocene. During the Neolithic Revolution and the transition from hunter-gatherer to farmer societies, followed by civilization changes in the Antiquity period, the dog population structure also changed. This process was due to replacement with newly formed dog breeds.

Methods

In this study we have presented for the first time mitochondrial data about South-Eastern Europe (the Balkans) ancient dog remains from the Early Neolithic (8 000 years BP) to the Late Antiquity ages (up to 3th century AD). A total of 25 samples were analyzed using the mitochondrial D-loop region (HVR1).

Results

The results have shown the presence of A (70%) and B (25%) clades throughout the whole investigated period. In order to clarify the position of our results within the ancient dog population in Eneolithic Eurasia, we performed phylogenetic analysis with the available genetic data sets. This data revealed a similarity of the Bulgarian dogs' structure to that of ancient Italian dogs (A, B, and C clades), which suggests a new prehistoric and historic Mediterranean dog population. A clear border can be seen between South-European genetic dog structure, on the one hand, and, on the other hand, Central-West (clade C), East (clade D) and North Europe (clades A and C). This corresponds to genetic data for European humans during the same period without admixture between dog populations.

Conclusions

49 Our data have shown for the first time the presence of clade B in ancient Eurasia. This is not
50 unexpected as the B haplogroup is widely distributed in extant Balkan dogs and wolves. The
51 presence of this clade both in dogs and in wolves on the Balkans may be explained with
52 hybridization events before the Neolithic period. The spreading of this clade across Europe
53 together with the A clade is related to the possible dissemination of newly formed dog breeds
54 from Ancient Greece, Thrace and the Roman Empire.

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56 **Key words:** ancient DNA, population structure, dog, the Balkans

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Introduction

59 In recent years, it has been increasingly assumed that the dog's domestication was a very
60 early process that began at the end of the Pleistocene. The dog's domestication is apparently
61 linked to the gradual synanthropy of wolf populations as a result of commensal relations
62 between man and wolf at the end of the late Pleistocene [1,2]. The first data from dog-like
63 remains came from the Razboinichya Cave in the Altai Mountains of Siberia (33 000 years BP)
64 and the Goyet cave in Belgium (c. 31 700 BP) [3,4]. Genetic studies of these remains have not
65 shown any similarity to recent wolves and dogs [3,5]. The Pleistocene domestication cannot be
66 accepted as absolutely certain, given the large morphological variability of Pleistocene wolves
67 [6].

68 Therefore, we can assert that signs of safe substitution are found only in the borderline
69 between the Paleolithic and the Mesolithic. Since then, the main questions have been related to
70 the place and time of origin, domestication and influence of hybridization events between
71 domesticated dog and local wolf populations.

72 According to mitochondrial DNA (mtDNA) studies, there are six main clades, assigned A,
73 B, C, D, E, and F, and many sub-clades, characterizing dog population in the world [7-9]. Clades
74 A, B, and C are the most widely distributed (95.9%) among recent dog populations, while D,
75 E, and F have regional geographic distribution [10]. For example, in West Eurasia, the sub-
76 haplogroup A1 is with a frequency of about 70%, while clades B and C are present with
77 frequencies of 20% and 10%, respectively [7,9]. In the Middle East, this proportion is almost
78 the same but with more worldwide presented sub-haplogroups [11].

79 Analysis of ancient dog DNA is still scarce and does not include many geographic regions.
80 Despite this, available data reveal quite different population structure concerning present day
81 dogs. These alterations are due to the emergence of the first civilizations as well as the early
82 historical and modern human migration. For example, pre-Columbian American dogs were

83 identical to East-Eurasian (Siberian) dogs which are nowadays mixed with West-Eurasian dogs,
84 after the Age of Discovery (15th century) [5,12,13]. Similarly, in the Pacific, including the
85 islands of Polynesia, a post-Lapita dog introduction from southern Island Southeast Asia has
86 been suggested [14].

87 **Ancient dog populations from Europe**

88 One of the most investigated regions concerning ancient dog mtDNA is Europe. Up to date,
89 most studies have shown that old Europe (from the Pleistocene to the Holocene) may be divided
90 into at least three or four regions of dog populations based on mtDNA analysis. One of them is
91 Central and West Europe, including France, Germany, Switzerland, and Hungary. Typical for
92 these parts of Europe is the prevalence of clade C (over 80%) mixed with clade D [5,13,15,16]
93 (Fig 1a). The second region includes Eastern Europe (Romania, Moldova, Ukraine) up to Iran
94 and the Middle East. The basic clade in these regions is D (over 90%), and there are traces of
95 A and C [5,13,16,17]. In Northern Europe (Scandinavia and Estonia) there are clades A and C
96 [5,16,18]. Also, the most presentable nowadays clade D is missing [7,19,20]. There has been
97 only one study from the Mediterranean region concerning ancient dog populations [21]. This
98 research includes only five samples (three wolves and two dogs), and the obtained results have
99 shown the presence of clades A, B, and C [5,21]. Most of the authors have suggested that clade
100 A is associated with Neolithic farmer migration in ancient Europe from the Middle East, while
101 clade B probably originated from Southeastern Europe (the Balkans and the Apennines)
102 because of the high frequency of extant Balkan wolves with similar haplotypes [5,21-24].

103 All these data from pre-historic Europe have shown a dramatic difference between ancient
104 and present day dog populations. This change may be explained with the replacement of local
105 dog breeds with more improved breeds from the South part of Europe, the Mediterranean
106 region, and the Near East during the Late Antiquity and the Medieval ages [21].

107 **Ancient dog populations in Central Asia and pre-Columbian**

108 **Americas**

109 Another well studied area of research is related to the region of pre-Columbian America and
110 the migration of Paleolithic hunter-gatherers and their dogs from Siberia via the North Asian
111 Mammoth steppe via the Beringia land bridge roughly 14 000 years ago [25,26]. MtDNA
112 studies of ancient dogs from Siberia and the Americas reveal a similar genetic profile, i.e. the
113 presence of specific A1 and A2 subclades [5,12,16,27,28] (Fig 1a). A similar mtDNA profile
114 with a prevalence of clade A has been found in ancient dogs from Turkmenistan (Central Asia)
115 [16].

116 **Ancient dog populations in South-Eastern Asia and the Pacific**

117 The third most studied geographic regions are South-Eastern Asia and the Pacific Islands,
118 including Australia (Fig 1a). Characteristic of these regions is the Neolithic pottery Lapita
119 culture, where a Pacific Ocean people flourished in the Pacific Islands from about 3 600 BP to
120 about 2 500 BP from continental Asia. A basic characteristic of dog populations in this region
121 is the prevalence of sub-clade A2 and a few cases of B and A4's [13,14,29]. These sub-
122 haplogroups are typical for recent South-Eastern Asia but with different frequencies [7,8].
123 Interestingly, the sub-clade B2 is different from West Eurasian dog's B1 myotypes [9].

124 In conclusion, dogs are a very interesting object, because of the direct evidence of historical
125 human migration processes. However, there are still many uninvestigated geographic regions.
126 White spots of ancient dog genetic diversity are, for example, the Mediterranean region,
127 including South Europe, the Near East, and the Fertile Crescent, and also other domesticated
128 centers in Central and East Asia (China and India), etc.

129 Our study tries to enrich data for ancient South-Eastern Europe – the Balkan Peninsula
130 (Bulgarian) dogs, based on mtDNA (partial D-loop region, HVR1) analysis. We have

131 performed phylogenetic analysis to compare our results with other ancient and recent European
132 dog and wolf populations.

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Results

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Phylogenetic analysis and haplogroup classification

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Ancient DNA was successfully amplified in 21 out of 25 samples tested. Out of the 21 sequences, 16 were used for phylogenetic analysis, because only they covered informative sites for haplogroups assignment. From them, eleven sequences were assigned to clade A (68.7%), four belonged to clade B (25.0%), and one to clade D (6.2%). These sequences have informative sites from position 15 595 bp to 15784 bp (189 bp) of the first amplified region of HVR1 according to ref. sequence EU789787 [8]. We analyzed only this region, because most of the ancient dog sequences available in GenBank also used this region as the most informative one for haplogroups assignment. Four of our not assigned sequences have only the third amplified fragment of HVR1, which is not declarative concerning haplogroup assignment (S1Table).

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All analyzed sequences in this study belonging to clade A and B are equally dispersed in investigated regions in Bulgaria as well as in historical time. In contrast, the highly dispersed in ancient East Europe clade D was found only in one sample from the Late Antiquity period (S1 Table).

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Comparative analyses of ancient dog haplotypes and Bulgarian

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samples

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About 230 ancient dog sequences available in GenBank and Dryad Digital Repository package [17,40] were explored by Reduce-median network analysis. In this analysis, ancient dog samples were from ancient Eurasia, pre-Columbian America and Pacific pre-Lapita period

154 (Fig 1b; S1 Table). All haplotypes were separated into four main clades: A, B, C and D. There
155 is a clear geographical differentiation between the distribution of C and D clades, which are
156 specific for Central, West and East Europe. Clade A is split into many sub-clades, i.e. A2 for
157 South-Eastern Asia and the Pacific, A1 for Siberia and Central Asia, and pre-Columbian
158 America, A4 and A6 for North Europe (Scandinavia), and A and B clade are characteristic for
159 the Mediterranean region (Italy, Bulgaria and Israel). Similarly, PCA analysis grouped all
160 ancient samples into five distinct clades (Fig 1c).

161 **Comparative analysis among ancient and recent Balkan dogs as** 162 **well as recent gray wolf haplotypes distribution**

163 About 120 haplotypes including recent Balkan dogs and wolves populations as well as
164 ancient Bulgarian and Italian samples were analyzed by Median-joining network (S1 Fig). All
165 samples (dogs and wolves) were explored as described previously from position 15 595 bp to
166 15784 bp (189 bp) of the first amplified region of HVR1 according to ref. sequence EU789787
167 [8], and haplotypes were determined according to the classifications of [9] and [34] as well as
168 MitoToolPy program [35]. The results show that most of the ancient and recent dog haplotypes
169 from clades A and B have similar or identical to recent Balkan wolves haplotypes. The Balkan
170 wolves haplotypes are not grouped into C and D clades. Moreover, there are wolves haplotypes
171 which not include present-day dog haplotypes.

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173 **Discussion**

174 **Balkan Neolithic and Chalcolithic periods: specifics of human**
175 **societies and understanding of early European farmer migrations**
176 **ways from the Near East.**

177 The South-Eastern Europe includes the Balkan Peninsula and the Apennines. These
178 geographical regions are important points concerning human history and migration processes
179 as well as the accompanying domesticated animals and the crops from the Near East. It is well
180 known that during the Last Glacial Maximum (22 000–14 000 BP) the sea level of the World
181 Ocean was about 125 meters lower than nowadays [41]. Therefore, large territories of land were
182 connected. For example, the Balkan Peninsula was connected terrestrially to Anatolia via the
183 Sea of Marmara in the east and also to a large part of the Apennines via the Adriatic Sea in the
184 west [21]. This enormous contact zone was a precondition for the free movement of human
185 societies but also of wild animal populations during the Mesolithic the and Early Neolithic
186 periods.

187 Therefore, Early Neolithic cultures from the Fertile Crescent and Anatolia were directly
188 connected to South-Eastern Europe (especially during the Late Neolithic period 8 000 BP).
189 During this period three migration ways for dissemination of Neolithic farmers into Europe
190 existed – the Mediterranean, the Balkans (the Danube River) and the North Pontic steppes (Fig
191 1a).

192 The most recent study on human population from the Mesolithic to the Iron Age has revealed
193 that human populations on the Balkans (South-Eastern Europe) and in Anatolia shared a similar
194 genetic profile in contrast with North Pontic Steppe Neolithic humans and those in Central and
195 West Europe [42]. Despite the similarity of the Eastern and the South-Eastern Neolithic and
196 Chalcolithic cultures, these genetic data suggest different migration routes (the Mediterranean,
197 the Danubean and the North Pontic) of farmers from the Fertile Crescent. The data of this
198 research clearly show human migrations from North to East direction on the Balkans during the
199 Chalcolithic and the Bronze Ages, but also dissemination of South-Eastern human farmers into
200 the Central Europe region (LBK culture – Austria, 5100–5000 BC). This evidence also suggests
201 different migration routes for livestock like cattle, goats, and sheep [35,43].

202 In the Neolithic to the Chalcolithic Age livestock farming on the Balkans was related related
203 to intensive cattle, goats and sheep breeding [31,44,45], with a prevalence of cattle (over 30%)
204 in the North Balkans and domination of sheep breeding in the south. This animal husbandry
205 was accompanied everywhere by the presence in the studied settlements of ancient races of
206 dogs, which in later epochs passed into specially selected breeds.

207 The processes of domestication and dog breed creation continued later up to the Early and
208 the Late Antique periods. At this time the first data on dog breeds on the Balkans were described
209 by Aristoteles [46] and later, in the Roman empire, by Xenophon (Cynegeneticus, 2,000 years
210 ago). The authors commented on dozens of different dog breeds, including guard, hunting, and
211 companion dogs.

212 **Population structure of ancient dogs from the Neolithic period**

213 The question of dog origin is geographically, genetically, and archaeologically complex.
214 Ancient DNA analysis from relevant areas of the world should allow better understanding of
215 the evolution of dogs from their predecessor, the gray wolf. In this respect, there are a few
216 hypotheses about dog domestication and spreading. Based on recent mtDNA haplotypes
217 distribution, the common opinion is that in the center of domestication the genetic diversity is
218 the highest, thus there must be a high level of various haplotypes from different clades. Due to
219 this reason, the East Asian and the Near East (Anatolian and Fertile Crescent) centers were
220 proposed for dog origin centers [8,11]. The migration routes from the centers of domestication
221 are characterized by a bottleneck type of dissemination which is unique for bordered regions
222 worldwide like Europe, North Africa, Pacific Islands etc. For example, the genetic profile of
223 recent European dogs is characterized by the prevalence with various A1 and B1 sub-clades
224 [9,47], while dogs in South-West Asia (Anatolia and Fertile Crescent) have a mixed profile
225 with other A and B sub-clades [11]. Recent European C1, D1 and D2 subclades are considered

226 as regional specific sub-clades and as a “relict” from ancient time for dog-wolf hybridization
227 [7,8].

228 In contrast to molecular data about recent dogs, evidence for ancient dogs, though still
229 scarce, showed homogeneous and simple genetic structure of prehistory dog populations (Fig.
230 1a). In West, Central and North Europe there is a prevalence of C clade, in East Europe and the
231 Middle East (Iran) – of clade D, in Southeastern Asia, Australia, and the Pacific Islands – a
232 prevalence of the specific sub-clade A2, and in Siberia, Central Asia (Turkmenistan), and pre-
233 Columbian America – of specific A1 sub-clades [5,12-16,27,28] (Fig 1b). Key geographic
234 regions for understanding dog history like South Europe and the Near East, North Africa, and
235 ancient Central and South Asia (China and India) are still uninvestigated.

236 The prehistoric Neolithic Balkan dogs were primitive in terms of stage of domestication, as
237 their morphological features were relatively close to the ancestral wolf morphology as the
238 “*vlasac*” type [30,31,48]. Our data have shown a prevalence of dog A and B clades from the
239 Neolithic to the Antique period. During this time, the proportion of these clades was preserved
240 with minor changes. Despite the small size of the investigated samples, these results are in
241 direct contrast with the border regions in the north direction where the Danube River serves as
242 a border region. The investigated ancient dog samples from Romania, Moldova, and Ukraine
243 (9 000 – 5 000 BP) showed a prevalence of C and D clades [17], (S1 Table). Otherwise, our
244 data correlate with ancient samples from Italy and Israel, where, despite the small size of
245 samples, a prevalence of clades A and C for Italy has been revealed [5,16,17,21]. These data,
246 even insufficient, have suggested that A clade dog populations replaced old Central European
247 ones. It is possible that this genetic profile is associated with the Fertile Crescent and Anatolian
248 ancient dogs.

249 **Clade B, a possibility of South-Eastern European (Balkans) origin**
250 **of dogs from local wolves predecessors.**

251 In modern dogs, clade B is widely distributed with a frequency of over 20% [7,9]. This clade
252 consists of two sub-clades, B1 and B2. The B1 sub-clade is disseminated worldwide with a
253 frequency of about 21%, while the B2 sub-clade is with regional distribution mainly in Eastern
254 Asia with a frequency of about 10 % [9,47]. Although there is a high frequency of clade B in
255 modern dogs, in ancient samples up to date, these subhaplogroups have been observed very
256 rarely, about 1 %. There is one sample in France and Turkmenistan, and there are a few samples
257 from South-Eastern Asia and Oceania [14,16,17]. In our sample sets B1 sub-clade is with a high
258 frequency (over 20%). This haplogroup is common among the Neolithic, the Chalcolithic and
259 the Bronze Age samples but is also present in the Antique period with almost equal frequency.

260 These data are not very surprising because clade B is widely and unusually distributed in
261 recent Balkan wolf population, identified in several researches [5,22-24,37]. These studies
262 proposed that clade B possibly originated from the Balkans, although there are some cases of
263 B type wolves worldwide. In a previous study of Bulgarian native dogs, clade B was observed
264 with about 20 % [38].

265 The only investigation concerning South Europe about ancient dogs and wolves has been
266 carried out by [21 In this study they investigated three wolves or large canids from 14 000 – 10
267 000 years ago and two medium size dogs from 4 000 years ago. One of the wolves' samples
268 was assigned as clade B (PIC-2), while others belonged to A (PIC-3) and C (PIC-1) clades (S1
269 Fig). The authors hypotesized about the possibile origin of B clades from the Balkans due to
270 the high frequency of B type Balkan wolves. In addition, the authors defined that in South-
271 Eastern Europe (the Balkans and the Apennines) there is a high frequency of clade B in gray
272 wolf population. The discovery of clade B in the primitive dog race (intermedius-vlasac
273 morpho-group) from the Early Neolithic of Slatina (Sofia) presented new arguments in favor

274 of the morphologically [30,48] and genetically [21] argued hypothesis that some
275 prehistoric to recent dog races (races which have the B-clade) originated on the Balkans.

276 Taken together, all data about ancient dogs' populations up to now, even though partly
277 insufficient, have revealed a massive replacement of ancient local dogs before the Antique
278 period and nowadays. These processes may easily be explained with the creation of new dog
279 breeds in South Europe during the Antique and the Roman Empire periods, which disseminated
280 and replaced local dogs in north direction in Medieval Europe. A similar process was also
281 observed in different time and geographic regions during the Ages of Discovery, where C and
282 B sub-clades changed the genetic structure of local American dogs via the introduction of extant
283 European dog breeds.

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Conclusion

286 In conclusion, our data have enriched the information about ancient dogs' structure,
287 especially in South-Eastern Europe. The results from the Neolithic, the Chalcolithic and the
288 Antique periods in Bulgaria, have demonstrated the dominance of A (70%) and B (25%) dog
289 clades and homogenic structures of these haplogroups throughout the whole investigated period
290 but an absence of clade C and only one case of D clade (late Antique period). These data have
291 revealed the similarity of the Bulgarian dogs' structure to that of ancient Italian dogs (A, B and
292 C clades). Also, our data have shown for the first time the presence of clade B in ancient Eurasia.
293 This is not unexpected because of the fact that the B haplogroup was widely distributed in extant
294 Balkan wolves and dogs. The presence of this clade both in wolves and in dogs on the Balkans
295 may be explained with hybridization events before the Neolithic period. Spreading of this clade
296 across Europe together with the A clade is related to possible dispersal dissemination of newly
297 formed dog breeds from Ancient Greece, Thrace and the Roman Empire.

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Materials and Methods

301 **Archeological samples collection, ancient DNA isolation and PCR**

302 **amplification and sequencing**

303 Twenty-five samples (bones and dental material) from ancient dogs from the collection of
304 NMNH-BAS were studied from several archaeological sites: Early Neolithic 8500-7500 BP
305 (Gradeshnitsa/Malo Pole, Ohoden–Valoga Slatina, Sofia district); Late Neolithic 7500-7000
306 BP (Topolnica, Promachon and Budzhaka-Sozopol); Early Chalcolithic 6950-6500 BP (Okol-
307 Glava, Gniljane, Sultan (Nevski) Popovo, and Settlement mound Burgas); Late Chalcolithic
308 6500-6000 BP (Dolnoslav, Varna); Bronze 6000-5000 BP (Urdoviza – Kiten, Baley); Late
309 antiquity (Kapitan Andreevo, Dyadovo village – Nova Zagora, Charda village, Yambol district,
310 Trakia motorway, Yambol district, and Academic, Plovdiv) (S1 Table and S1 Fig).

311 The *Canis familiaris* remains were determined and assigned to the two primitive prehistoric
312 morphotypes (and probable crossbreeds between them), representing the two main
313 Mesolithic\Neolithic-Chalcolithic canine races of the Balkans that can be called conditionally
314 *Canis familiaris* “*intermedius*” and *C. f.* “*palustris*”. The mesolithic form “*vlasac*”, described
315 from Serbia [30] is not different from *C. f. intermedius* and represents a slightly younger form
316 of the latter, with skull/tooth dimensions that are in general within the lower values of the
317 individual variations of *C. f. vlasac*, while “*C. f. palustris*” is the more domesticated small form
318 [31].

319 Ancient DNA isolation and PCR amplification and sequencing were given in the Supporting
320 Information S1.

321

322 **Phylogenetic reconstruction**

323 The obtained sequences were manually edited and aligned by MEGA software version 7.0³²,
324 using the dog mtDNA sequence NC_002008 [33] and EU789787 [8] as a reference. Sequences
325 were analyzed by polymorphic SNPs, and haplogroups were determined according to [9] and
326 [34] as well as MitoToolPy program [35], (<http://www.mitotool.org/mp.html>) with reference
327 sequence EU789787 [8]. The phylogenetic analysis was based on the archaeological dog
328 samples used in this study as well as on all available in GenBank ancient DNA dog sequences
329 [5,12-14,16-18,21,27-29,36]. Ancient and recent wolves [5,21-24,37], and the recent Bulgarian
330 native dog [38] were characterized using network analysis – NETWORK 4.5.1.6 (Fluxus
331 Technology Ltd.) (available at <http://fluxusengineering.com>).

332 In order to graphically display (and summarize) the mitochondrial relationships among the
333 analyzed ancient dog populations and all ancient samples available in GenBank, we performed
334 a principal component analysis (PCA) – a method that considers each haplogroup as a discrete
335 variable and allows a summary of the initial dataset into principal components (PCs). Principal
336 component analyses (PCA) were performed using Excel software implemented by XLSTAT,
337 as described elsewhere [39]. The PCA were carried out considering all available ancient dog
338 sample worldwide.

339 The obtained sequences included in this study were deposited in the National Center for
340 Biotechnology Information (NCBI) GenBank database under accession numbers NCBI:
341 MH937186–MH937206.

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344 **Data availability statement**

345 Consensus sequences from this study are available from the GenBank database (accession
346 numbers MH937186–MH937206).

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349 **Author Contributions**

350 M.M., G.R., and P.H. conceived of and designed the experiments; G.R., P.H., and N.S. wrote
351 the manuscript text; I.Y. and M.P. performed the experiments; G.R., P.H., M.M., and N.S.
352 analyzed the data.

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354 **Competing interests**

355 The authors declare no competing interests.

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512 Figure caption

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514 **Figure 1.** Population structure of ancient dogs worldwide based on mtDNA data.

515 a) A world map showing distribution of main haplogroups in ancient dog. The main migration
516 routes are also shown. b) The reduced median network of the main mtDNA haplotypes from
517 ancient dogs of different regions of the world, c) Principal components analysis of the main
518 mtDNA haplotypes from ancient dogs of different regions of the world

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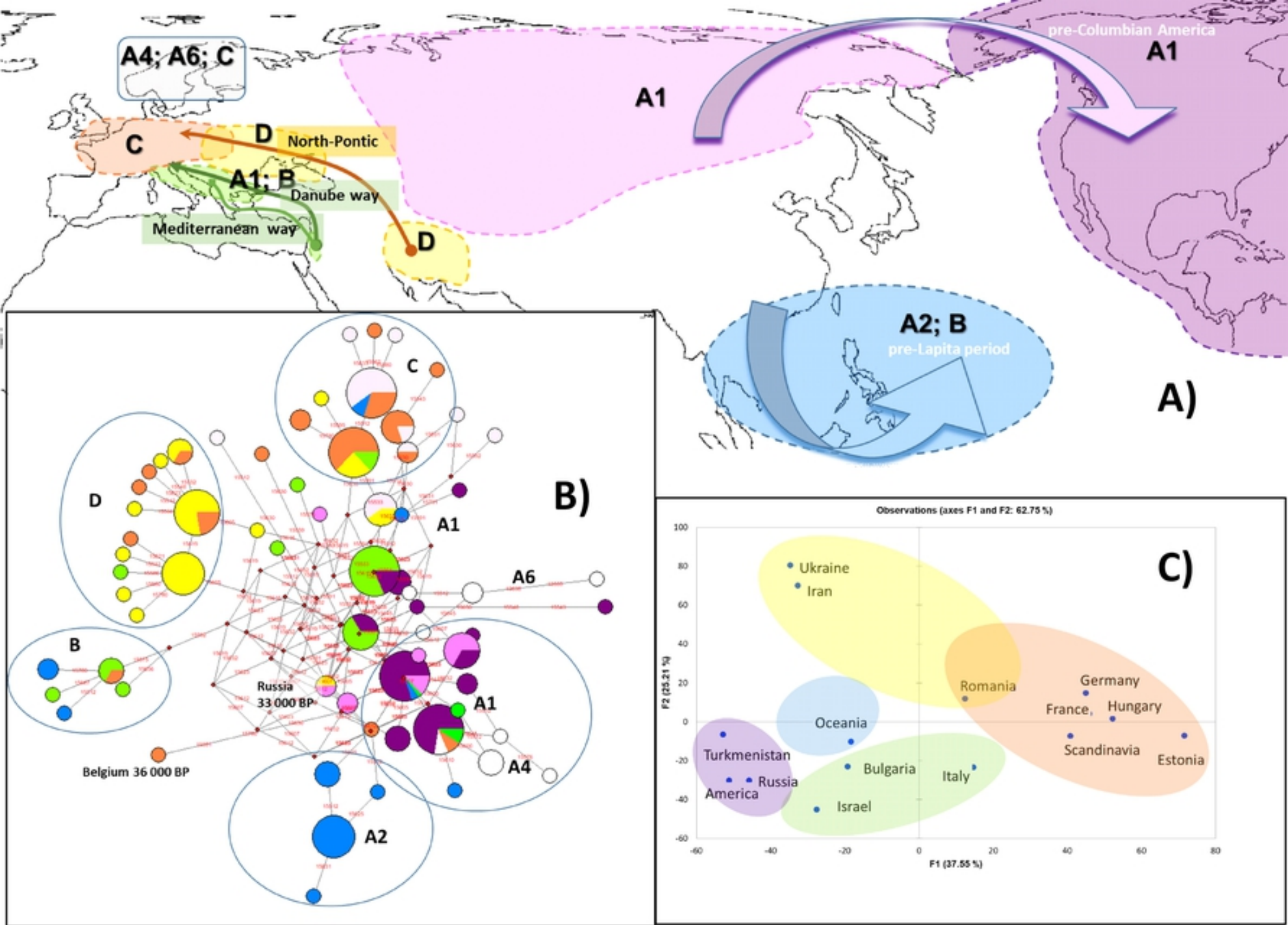


Figure 1