



The role of the Caucasus, Carpathian, and Dinaric–Balkan regions in preserving wolf genetic diversity

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Abstract

Mountain regions have long been important for maintaining populations and genetic diversity of wild species, especially those species that require large areas to sustain viable populations. We examined wolves (*Canis lupus*) in the Caucasus, Carpathian, and Dinaric–Balkan regions, expecting these persistent populations to contain high genetic diversity and an overlap of the major haplogroups detected in earlier broad-scale investigations. We analyzed 926 mitochondrial DNA control region sequences, including 533 new samples whose geographic distribution allowed us to reduce sampling gaps observed in previous broad-scale studies. We estimated genetic variability, population structure, and phylogeographic relationships to evaluate the diversity and connectivity of populations throughout the study regions.

We detected haplogroups H1 and H2 that overlapped across the study regions. Haplogroup H1 can be divided into three subgroups: H1A and H1B that partially overlap throughout the study regions, and H1C that was found only in wolves from Armenia. Haplogroup H2 was largely confined to the Carpathian and Dinaric–Balkan regions. Our analyses of population structure partly concurred with the haplogroup distribution and produced four major genetic clusters. Our results demonstrated high genetic diversity within the study regions, supporting their role in maintaining intraspecific variability in wolves and other species that require large areas to sustain viable populations. The unique diversity and north–south structure observed within the Caucasus emphasize the need for further research and conservation efforts in this highly biodiverse region. Our findings highlight the role of broad-scale planning in conserving evolutionary processes in this and other transboundary areas.

Keywords *Canis lupus* · Genetic diversity · Mitochondrial DNA · Transboundary regions

Introduction

The grey wolf (*Canis lupus*), historically widespread in Europe, experienced a decline in its distribution in the mid twentieth century, mainly due to various human activities, including overhunting, poaching, and habitat fragmentation (e.g., Mech and Boitani 2007; Dufresnes et al. 2018; Musto et al. 2021). These activities have been particularly detrimental to smaller, isolated populations, and those living in close proximity to humans (e.g., Chapron et al. 2014;

Nowak et al. 2021). As a result, the distribution and size of some populations declined drastically until the end of the twentieth century (e.g., the Central European population, Andersen et al. 2015, Reinhardt et al. 2015; the Italian population, Lucchini et al. 2004; Fabbri et al. 2007; the Baltic population, Jędrzejewski et al. 2005), whereas some eventually became extinct (e.g., the Sierra Morena population, López-Bao et al. 2015). Consequently, the genetic diversity of these populations declined or was permanently lost, which is expected to negatively affect the adaptive potential of the species (e.g., Frankham 2005). Populations in areas where wild habitats, including mountains, are more abundant, such as the Carpathian Mountains (hereafter Carpathians), the Dinaric Mountains (hereafter Dinarics, as described in Šnjegota et al. 2021), and the Balkan Mountains (hereafter Balkans, as outlined in Djan et al. 2014), were less exposed

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to the negative impact of human activity and remained more stable over time (e.g., Filonov 1989; Okarma 1993). These regions and their populations have been reservoirs of genetic diversity for the recent remarkable recolonization of Europe by wolves (Chapron et al. 2014; Gula et al. 2009; Ražen et al. 2016; Nowak et al. 2017).

The Carpathian and Dinaric–Balkan populations have also been considered as potential south–north and east–west links between wolf populations (e.g., Stronen et al. 2013; Pilot et al. 2014), due to their central position in Europe. Eastern European wolves were shown to be connected with Western Asian wolves via the Caucasus region (Pilot et al. 2014). Despite the important role of the Caucasus as a trans-boundary region, population genetic studies on wolves from this region have been sporadic to date (e.g., Pilot et al. 2014; Kopaliani et al. 2014; Shakarashvili et al. 2020). Conversely, there have been several population genetic studies on wolves from the Carpathians, Dinarics, and Balkans, although most are characterized by a discontinuous or limited sampling area. In the Carpathians, for example, population genetic studies have mainly included the northern mountainous regions (Pilot et al. 2006, 2010; Gula et al. 2009; Czarnomska et al. 2013; Bakan et al. 2014; Rigg et al. 2014), whereas in the Balkans, study areas have been largely fragmented (Pilot et al. 2010; Gomerčić et al. 2010; Djan et al. 2014; Stronen et al. 2013; Šnjegota 2019).

For a highly mobile species, we expect that more continuous and extensive sampling from persistent populations will demonstrate high genetic diversity and overlap in the haplogroups reported in previous studies. We sampled wolves from the Caucasus region in the east, through the Ukrainian steppes to the Carpathians, and southward through the Dinarics and Balkans. We analyzed (i) genetic diversity, (ii) population structure, and (iii) phylogeographic relationships of wolves from these regions, to better understand their connectivity and role in maintaining genetic diversity.

Materials and methods

Study area and sampling

Wolves were sampled during 1998–2018, between 13°–50°E longitude and 33°–50°N latitude, an area spanning about 2800 km east–west and 1300 km north–south (Fig. 1, Appendix Table A1), in the Carpathians in the north, the Dinaric–Balkans in the southwest, and the Caucasus in the east (more details about the study area are provided in Appendix Note A1). The maximum altitudes varied from 1500 to 2925 m above sea level (a.s.l.) in the south-western part to 5642 m a.s.l. in the Greater Caucasus Mountains at the northern shore of the Black Sea. Between Caucasus and

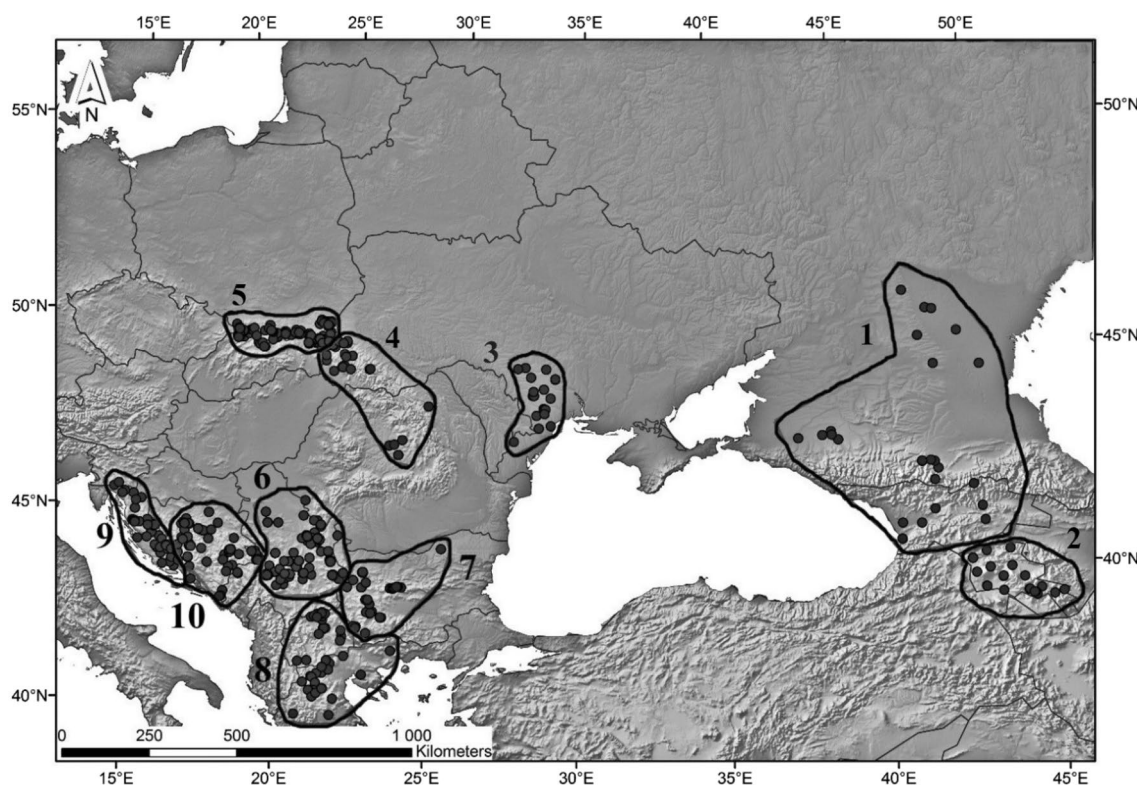


Fig. 1 Wolf sampling sites labeled according to Table 1

Carpathians are lowlands of Ciscaucasia, the Black Sea Lowland, and the Volyn-Podolian Upland. We collected samples also in the Black Sea Lowland region, located almost completely within southern Ukraine which is characterized by steppe landscape and accordingly named as the Ukrainian steppe in this study (more details about this study site are provided in Appendix Note A1). Our sampling included 14 countries in total: 4 in the Carpathians (Poland, Slovakia, Ukraine, and Romania), 7 in the Dinaric–Balkan region (Croatia, Serbia, Bosnia & Herzegovina (hereafter B&H), Montenegro, Bulgaria, North Macedonia, and Greece), and 3 countries within the Caucasus (Russia, Georgia, and Armenia).

The climate ranges from mountainous and temperate in the northern parts to Mediterranean in the south, and seasonality is well-pronounced throughout the study region. Mean daily temperatures depend on latitude and altitude, and they range from -12° to 10° °C in January and from 10° to 26° °C in July (European Environment Agency 2009a).

The mountain areas of the study regions, except for the southern part of the Lesser Caucasus, are highly forested (forests usually cover $> 50\%$, and often $> 75\%$ of the land surface), whereas plains and lowlands, used for farming, have forest cover below 25% (European Environment Agency 2009b). The major tree species in the forests of the Carpathian and Dinaric–Balkan regions are beech *Fagus sylvatica*, Norway spruce *Picea abies*, and silver fir *Abies alba*, whereas Mediterranean pine *Pinus* spp. forests grow in the southernmost regions (e.g., Greece). The Caucasus is characterized by mixed forests with domination of oriental beech *Fagus orientalis*, Caucasian spruce *Picea orientalis*, Caucasian fir *Abies nordmanniana*, and Caucasian elm *Zelkova caprinifolia* (Bohn et al. 2007).

The analyzed material included in total 533 samples collected from regular hunting and/or from road killed individuals, and noninvasive sampling of feces (Appendix Table A1). No animals were killed for the purpose of this study. After collection, samples were stored in 96% ethanol at -20° °C prior to DNA extraction. We supplemented our dataset with 393 additional grey wolf mtDNA control region sequences from our study regions downloaded from GenBank (Appendix Table A2).

Laboratory analyses

Isolation of DNA for samples from Serbia, B&H, and North Macedonia followed Djan et al. (2014). For the remaining samples, DNA isolation was conducted using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocols. A fragment of the mtDNA control region was amplified using the D3 and D4 primers (Savolainen et al. 1997) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). The total reaction volume ($10\text{ }\mu\text{l}$) contained: $5\text{ }\mu\text{l}$

of multiplex PCR mix; $0.5\text{ }\mu\text{l}$ of each primer; $2\text{ }\mu\text{l}$ of dH_2O ; and $2\text{ }\mu\text{l}$ of genomic DNA. Polymerase chain reaction (PCR) conditions were 95° °C for 15 min; 35 cycles of 94° °C for 15 s, 56° °C for 15 s, and 72° °C for 1 min; and 72° °C for 10 min. PCR products were purified using Clean Up (A&A Biotechnology, Gdańsk, Poland). Sequencing reactions were carried out in a $10\text{ }\mu\text{l}$ volume using the Big Dye sequencing kit v.3.1 (Applied Biosystems) with the forward primer. Products were purified with the Exterminator kit (A&A Biotechnology) and separated on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Sequencing results were analyzed with the ABI DNA Sequencing Analysis software, and manually checked in BioEdit v.7.7.0 (Hall 1999). The total length of sequences was 223 bp.

Statistical analyses

Statistical analyses were performed on the final dataset ($n = 926$ sequences), which was divided into ten study sites (Table 1). The basic parameters of mtDNA polymorphism (Na—the number of haplotypes, Hd—haplotype diversity, π —nucleotide diversity) were calculated in Arlequin v.3.5.2 (Excoffier and Lischer 2010), as well as pairwise Φ_{st} values between wolf populations from the study sites (Appendix Table A3) and genetic clusters detected by GENELAND (Appendix Table A4). Significance levels of pairwise Φ_{st} values were calculated using 10,000 permutations. The level of haplotype diversity was also evaluated by calculating the diversity index B (Levins 1968). The difference between B and Hd diversity indices is in the range of their minimum and maximum values; Hd varies from 0 to 1 whereas B varies from 1 to the maximum value which is equivalent to the number of haplotypes in the sample. Therefore, B can be interpreted as the effective number of haplotypes in the sample (Niedziałkowska et al. 2014).

Spatial analysis of molecular variance, implemented in the SAMOVA v.2.0 (Dupanloup et al. 2002), was used for detecting population genetic structure. This method allows identification of subpopulations that are geographically homogeneous and differentiated from each other, by maximizing the proportion of total genetic variability (Φ_{CT}) due to differences between groups of populations. The analysis requires a priori definition of K (the number of groups) and was run with K ranging from 2 to 10, following the program manual. The most probable number of groups was identified according to the mode of changes in Φ statistic parameter values. Additionally, population genetic structure was explored with GENELAND v.4.0.5 (Guillot et al. 2005) in the R 1.8.6. environment (R Core Team 2019), which also incorporates the spatial distribution of the samples. GENELAND was run with K ranging from 2 to 10; MCMC (Markov chain Monte Carlo) was run with 10^6 iterations and a thinning interval of 100 iterations, and by applying

Table 1 Molecular characteristics of wolves distributed throughout the ten sampling sites

No	Sampling regions and sites	<i>N</i>	<i>Na</i>	<i>Π</i>	<i>Hd</i>	<i>B</i>
The Caucasus						
1	Georgia, Russia	42	8	0.015	0.763	3.77
2	Armenia	33	5	0.019	0.716	3.27
	In total	75	6.5	0.017	0.739	3.52
3	Ukrainian steppe (in total)	27	6	0.019	0.553	3.17
The Carpathians						
4	Ukrainian and Romanian	50	6	0.013	0.709	2.25
5	Polish and Slovakian	228	9	0.022	0.665	3.01
	In total	279	7.5	0.017	0.687	2.63
The Balkans						
6	Serbia	181	9	0.015	0.508	2.01
7	Bulgaria	103	10	0.021	0.726	3.56
8	North Macedonia, Greece	71	9	0.019	0.614	2.53
	In total	355	9.3	0.018	0.616	2.7
The Dinarics						
9	Croatia	112	6	0.018	0.67	2.96
10	Bosnia & Herzegovina, Montenegro	79	5	0.019	0.727	3.54
	In total	191	5.5	0.019	0.698	3.25
In total		926	7.3	0.018	0.665	3.01

n number of individuals, *Na* number of haplotypes, *Π* nucleotide diversity, *Hd* haplotype diversity, *B* index of haplotype diversity

the correlated model of allele frequencies. The first 20% of the initial iterations were discarded as a burn-in period. The most probable *K* was chosen according to the posterior probability of population membership and the histogram of estimated *K* values for each run. The same parameters were used for the hierarchical analyses to explore the substructure in groups detected by GENELAND.

Phylogeographic relations among haplotypes were analyzed by creating a Median-Joining (MJ) network and a Bayesian phylogenetic tree. The network approach is more convenient than the tree approach as it allows the presence of ancestral haplotypes in a sample, whereas in the trees, all sequences are treated as terminal taxa (Posada and Crandall 2001). An MJ network was created in PopArt v.1.7 (Bandelt et al. 1999). The Bayesian tree was generated using BEAST 1.8.0 (Drummond and Rambaut 2007), and applying the HKY + G evolutionary model, suggested as the most appropriate model for our dataset by MEGA6 (Tamura et al. 2013). Constant population size was assumed as a coalescent tree prior, as, according to Drummond and Rambaut (2007), this is the most suitable for trees describing the relationships among individuals within the same population and/or species. The MCMC was run with 10^7 iterations and sampling every 10^4 generation. The first 10% of the initial trees in each run were discarded as burn-in periods. Tracer v.1.5 (Drummond and Rambaut 2007) was used for summarizing the results and for checking the convergence of all parameters. The tree was visualized using FigTree v.1.4.0 (Rambaut

2012) and rooted with a sequence from the coyote (*Canis latrans*, GenBank Accession no DQ480510.1).

Results

Genetic variability

After analyzing sequences of the wolf mtDNA control region with a total length of 223 bp, 26 mtDNA haplotypes were detected, including 5 new (i.e., not published in previous studies) haplotypes identified in single individuals from Serbia, B&H, Croatia, the Ukrainian steppe, and Russia (Appendix Table A2). The number of mtDNA haplotypes per sampling site ranged from 5 to 10 and the total number of polymorphic sites was 27 (7 singleton variable sites and 20 parsimony informative sites). The highest number of haplotypes was detected in Bulgaria (*Na* = 10), while the highest values of haplotype diversity *Hd* and *B* diversity indices were detected in the Caucasus (Russia and Georgia) (*Hd* = 0.76, *B* = 3.77). The lowest number of haplotypes was detected in B&H, Montenegro, and Armenia, whereas the lowest values of *Hd* and *B* diversity were recorded in Serbia (Table 1). Overall, the largest number of haplotypes was detected in the Balkans, followed by the Carpathians and the Caucasus, Ukrainian steppe and finally in the Dinarics. Haplotype diversity decreased from the Caucasus, followed

by the Dinarics and Balkans, to having the lowest values in the Carpathians (*B*) and Ukrainian steppe (*Hd*).

Population structure

Results from the SAMOVA and GENELAND analyses suggested four ($K=4$) genetic clusters (Fig. 2), which was supported with statistically significant pairwise Φ_{st} values (Appendix Table A4). In the SAMOVA analysis, the value of Φ_{CT} had the highest increase between $K=3$ and $K=4$, reaching its plateau at $K=4$ (Appendix Fig. A2). Therefore, $K=4$ seemed the most plausible number of clusters. The same number of clusters ($K=4$) was also suggested by the GENELAND analysis (Appendix Fig. A3), and we used these results to delineate four clusters, C1–C4. C1 included wolves from Croatia, B&H and Montenegro; C2 wolves from the Polish, Slovakian, Romanian, and Ukrainian Carpathians, the Ukrainian steppe, Serbia, Bulgaria, North Macedonia, and Greece; C3 wolves from Russia and Georgia, and group C4 comprised the wolves from Armenia (Fig. 2).

Given the results of previous studies in these regions (Pilot et al. 2006; Czarnomska et al. 2013; Stronen et al. 2013; Szewczyk et al. 2019), we investigated the possible presence of additional substructure within the large C2 cluster. GENELAND showed a division of the C2 cluster into the lowland Ukrainian steppe (C2A subcluster) and the mostly mountainous areas of the Carpathians and Balkans (C2B subcluster), with the border of the division running along the Carpathians (Fig. 2). The results of possible additional substructure of C2B subcluster were inconclusive and would require more detailed sampling.

The spatial distribution of the two detected haplogroups H1 and H2 partially concurred with the results

of SAMOVA and GENELAND (Figs. 3 and 4, Appendix Fig. A1). In haplogroup H1, three subgroups were defined: H1A, H1B, and H1C (Figs. 3 and 4, Appendix Fig. A1, Table A2). The spatial distribution of subgroups H1A and H1B included the entire study area, but with the predominance of different haplotypes in the various regions. For example, haplotypes w2, w7, and w3/78 dominated in the Carpathians, w77 in the Caucasus, w13 in the Balkans, w23 and w10 in the Dinarics, w4 and w7 in the Ukrainian steppe, and some of them overlapped throughout the study regions as predicted (Fig. 4, Appendix Table A2). Subgroups H1A and H1B had the broadest distribution in the Carpathians and Balkans, followed by the Caucasus, and lastly the Dinarics (Appendix Table A2). The subgroup H1C emerged as the most differentiated, with the haplotypes forming a monophyletic clade (Appendix Fig. A1), and these haplotypes were found only in Armenian wolves (Fig. 4).

Haplogroup H2 was detected across the study regions, dominating in the Carpathians and Balkans with the haplotype w6/14. This haplotype occurred sporadically or was completely absent in other regions (Fig. 4, Appendix Table A2). The second most frequent H2 haplotype w133 had the highest frequency in the Dinarics (Appendix Table A2). Haplotypes w9 and w11 were differentiated from the other haplotypes of this haplogroup, having distinct positions in the network (Fig. 3) and forming a monophyletic sister clade to the other haplotypes from haplogroup H2 (Appendix Fig. A1).

Fig. 2 Clusters ($K=4$) detected with SAMOVA and GENELAND based on analyses of wolf mtDNA control region haplotypes. The dashed line indicates the C2A subcluster, while all other samples from C2 cluster belong to the C2B subcluster, according to the hierarchical analysis of this cluster

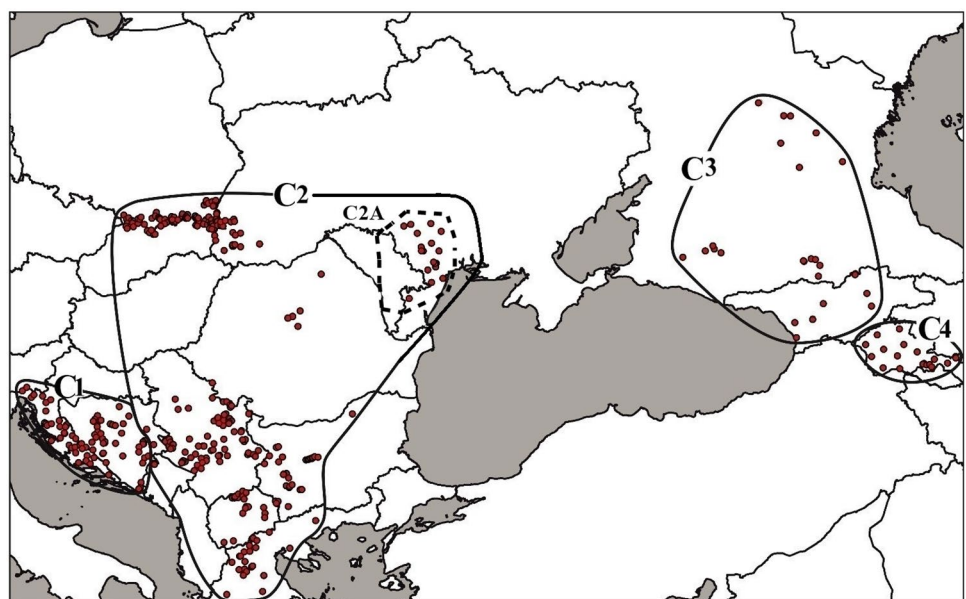
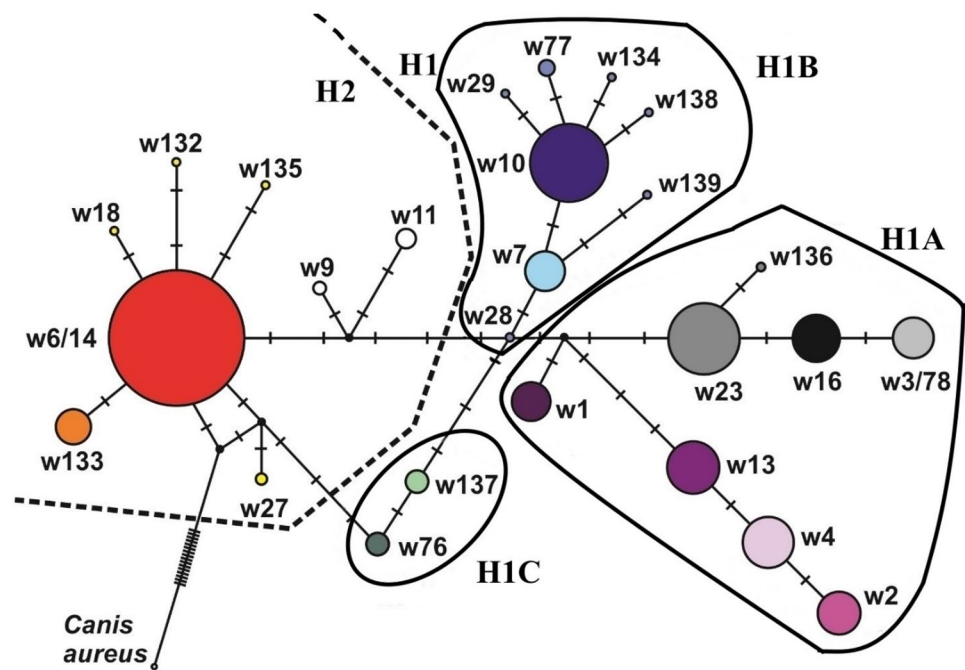


Fig. 3 A network showing phylogenetic relationships among wolf mtDNA control region haplotypes, including the number of mutational steps. The size of the circles corresponds to the number of sequences per haplotype whereas the similarity in colors corresponds to the defined haplogroups H1A, H1B, H1C, and H2. The dashed line indicates the boundary between the two main haplogroups (H1 and H2)



Discussion

The distribution of haplogroups H1 and H2, which we detected throughout the study regions, partially coincided with the spatial distribution of the identified genetic clusters ($K=4$). Haplogroup H1 was divided into three subgroups: H1A, H1B, and H1C. Subgroups H1A and H1B overlapped throughout the study regions, whereas H1C was found only in the southern Caucasus, in wolves from Armenia. Haplogroup H2 was largely confined to the Carpathians and Balkans. Likewise, we observed corresponding regional structures in some places. Haplotype diversity decreased through the Caucasus, Dinarics, and Balkans, being at its lowest in the Carpathians (*B*) and Ukrainian steppe (*Hd*), respectively.

The Caucasian wolves: genetic differentiation between Armenia and Georgia

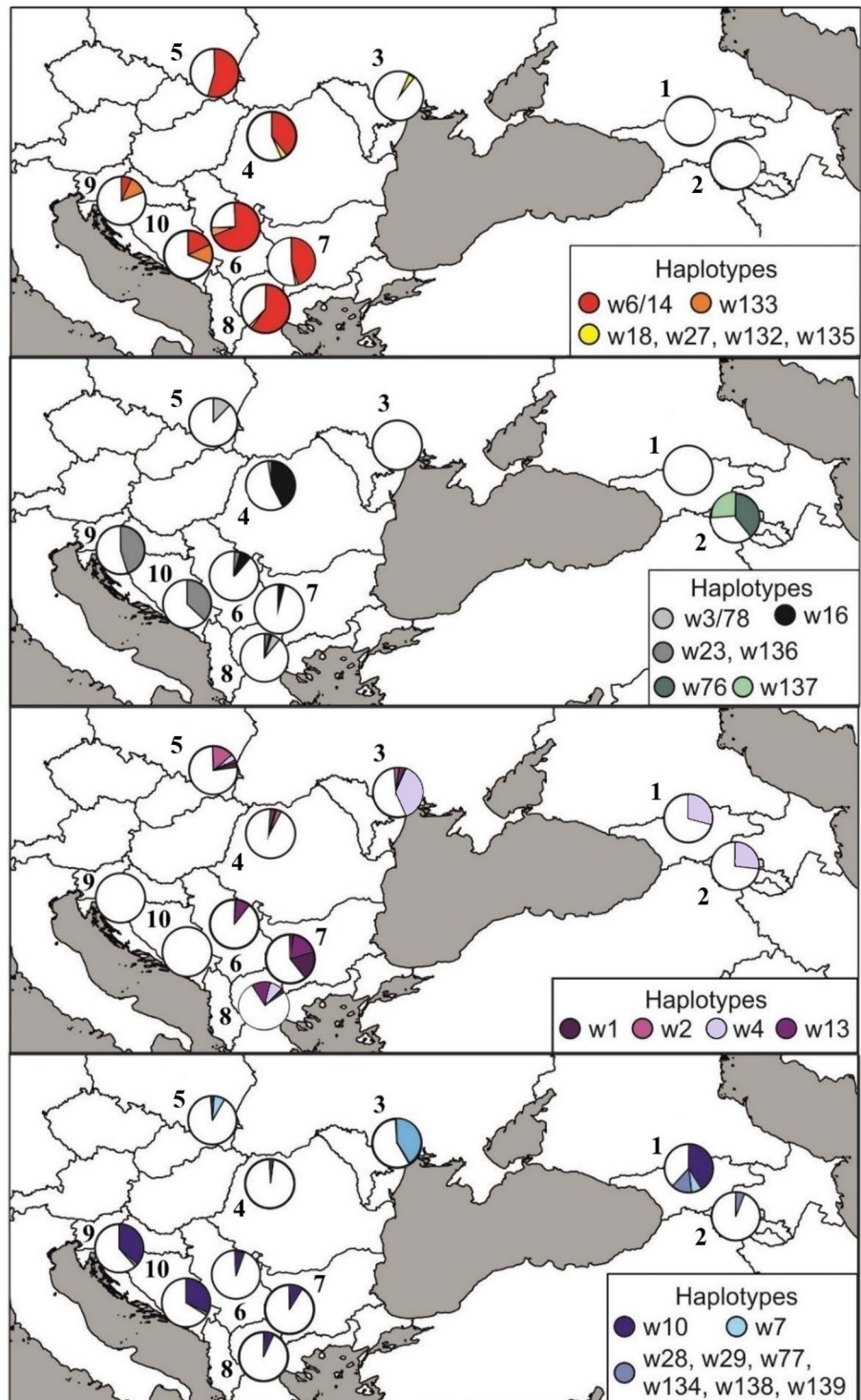
The Caucasian wolves showed the highest haplotype diversity (*B* and *Hd*). Despite having some haplotypes that overlapped between the Caucasus and the other regions (i.e., w4, w10), the newly detected haplotypes and those previously found only in the Caucasus (Pilot et al. 2014), indicated the presence of distinct variants in this region. The spatial distribution of the H1 and H2 haplotypes showed a moderate genetic division, whereas GENELAND revealed a pronounced differentiation within the Caucasian wolves, between Georgia and Armenia. Our results contrast with those of Pilot et al. (2014) who found no evidence of differentiation within the Caucasus, but our sampling sites were slightly different; both studies included wolves from

Georgia, whereas in southern Caucasus Pilot et al. (2014) included material from Nagorno-Karabakh, and we analyzed samples from Armenia. However, they accord with the results of Kopaliani et al. (2014), and Shakarashvili et al. (2020) who mentioned immigration from the east toward the west, and the possibility that wolves in the Caucasus including Armenia are connected to populations further east.

The contrasting conditions of the north and south Caucasus might affect the north–south gradient by directing movements of the Caucasian wolves in opposite directions, those from Georgia to the north and Armenian wolves to the south. Indeed, the contrasting climate and environmental conditions in different parts of the Caucasus were considered as factors affecting the north–south gradient visible in the population structures of other species inhabiting this region, including the white-breasted hedgehog (*Erinaceus concolor*; Seddon et al. 2002), the Alpine ibex and East Caucasian tur (*Capra ibex caucasica*, *C. cylindricornis*; Manceau et al. 1999), the Caucasian salamander (*Mertensiella caucasica*; Tarkhnishvili et al. 2012), the crested newt (*Triturus karelini*; Wallis and Arntzen 1989), the brown bear (*Ursus arctos*; Murtskhvaladze et al. 2010), many birds, butterflies, and reptiles (Harrison 1982; Higgins and Hargreaves 1983; Orth et al. 1996).

The Armenian wolves were distinct from those of Georgia, and from all other European wolves. The subgroup H1C, found only in Armenian wolves, was observed also in Nagorno-Karabakh (Pilot et al. 2014) and Turkey (İbiş et al. 2016). Presumably, H1C haplotypes extended toward the south and east, and their distribution range should be further explored. In Europe, the once dominant haplogroup

Fig. 4 Distribution of wolf mtDNA control region haplotypes throughout study sites, based on phylogenetic relationships of haplotypes as shown in Fig. 3. Haplotypes w9 and w11 are excluded due to their uncertain position in the network and low statistical significance in the Bayesian tree (Figure S1). Sampling sites are labeled according to Table 1



H2 was largely replaced by haplogroup H1 during the Holocene (Ciucani et al. 2019). During the Last Glacial Maximum (LGM), the Caucasus was better connected to Southern Europe, with Anatolia serving as a land bridge (Tzedakis et al. 1997; Aksuet et al. 1999). After the LGM, the connection between the Caucasus and Southeastern Europe was more tenuous due to postglacial sea-level-induced landscape

changes (Hewitt 2000). On the assumption that the H1C haplogroup has expanded over the last few thousand years in parallel with the expansion of the H1 haplogroup in Europe (Pilot et al. 2010; Ciucani et al. 2019) and North America (Leonard et al. 2007), the observed differentiation of Armenian wolves could be the result of limited gene flow between Southern Europe and Anatolia.

Studies of Caucasian hedgehogs suggested that the narrow band along the southern coast of the Black Sea (into northern Turkey), and the further regional extension toward the south-western Caucasus, represented a refugium for the southern population of this species (Seddon et al. 2002). A similar situation was also hypothesized for the presence of different *Quercus* species revealed by pollen records (Huntley 1990, 1992), reinforcing the idea of northern Turkey as a refugium for southern Caucasian lineages during the LGM.

The Carpathian–Balkan cluster

The Carpathians and Balkans showed slightly lower haplotype diversity than the other study regions. This is likely due to the intense and constant human persecutions that both populations have experienced (Hindrikson et al. 2016; Hulva et al. 2018), as well as substantial fragmentation of wolf habitat in the Carpathians during the last century (e.g., Babai and Molnár 2014; Butsic et al. 2017). The population size and distribution range of both Carpathian and Balkan wolf populations have subsequently decreased (e.g., Hell et al. 2001; Djan et al. 2014), but their continued existence has not been seriously compromised. Therefore, these populations have preserved a considerable amount of historical genetic diversity and have been more genetically diverse than other European populations (e.g., Pilot et al. 2010; Hindrikson et al. 2016). This relates especially to populations that were under high human pressure in the past, including lowland wolves (e.g., the Central European population, Andersen et al. 2015; Reinhardt et al. 2015; the Baltic population; Jędrzejewski et al. 2005) and isolated wolf populations (e.g., the Italian population, Lucchini et al. 2004; Fabbri et al. 2007; the Iberian population, Vilà et al. 1999). The Carpathian and Balkan populations have, thus, proved to be important reservoirs of historical genetic variability for neighboring populations, facilitating their recovery (e.g., Gula et al. 2009; Ražen et al. 2016).

The long-term persistence of wolves in the Carpathians and Balkans was also evidenced by the highest frequency of haplogroup H2 in both regions. This haplogroup is considered the evolutionarily oldest wolf haplogroup in Europe (Pilot et al. 2010) and its prevalence in the Carpathians and Balkans is not surprising, given the role of these regions as refugia during various environmental, geological, and anthropogenic events throughout history (Hewitt 2000; Schmitt 2009). The absence or occasional presence of this haplogroup elsewhere (Pilot et al. 2010) may suggest that the Carpathian and Balkan wolf populations are among the most persistent in Europe. The H1 haplogroup, subdivided into subgroups H1A and H1B, was less widespread in these regions and showed a north–south gradient, consistent with findings from single nucleotide polymorphism markers (Stronen et al. 2013).

Both the Carpathians and Balkans showed regional substructure in the west–east direction. In the Carpathians, we observed considerably higher haplotype diversity in wolves from the western Carpathians (Poland, Slovakia) than in the eastern Carpathians (Romania, Ukraine). The distribution of haplotypes suggested greater genetic similarity of wolves from the western Carpathians with those from the European lowlands (e.g., the Central European lowland population and wolves from the Ukrainian steppe) than with wolves from the Eastern Carpathians. This is expected given that the previous studies of wolves from the western Carpathians indicated a gradual (re)connection with neighboring lowland populations (Hulva et al. 2018; Szewczyk et al. 2019; Fehér et al. 2022). On the other hand, studies including wolves from the northern Carpathians indicated their isolation and differentiation from the wolves in the southern and southeastern Carpathians and lowland wolves in northern and northwestern Europe (Pilot et al. 2006, 2010; Gula et al. 2009; Czarnomska et al. 2013; Bakan et al. 2014; Rigg et al. 2014). Wolves in the eastern Carpathians showed higher genetic similarity with the Balkans (most notably Serbia) (Djan et al. 2014) and Turkey further east (Pilot et al. 2010; Randi et al. 2000; Montana et al. 2017) suggesting gene flow between the Carpathians and Balkans. Although previous studies of wolves indicated a strong differentiation between the Carpathians and Balkans (e.g., Stronen et al. 2013; Bakan et al. 2014), population genetic studies of other highly mobile species, such as the golden jackal (Rutkowski et al. 2015; Pyšková et al. 2016; Kowalczyk et al. 2020) and Carpathian lynx (e.g., Paunović et al. 2001) provide evidence of continued gene flow between these regions. However, it is important to note that the Carpathian–Balkan cluster observed in our study should be interpreted with caution. Recent research on whole mitogenomes of the domestic taurine cattle (*Bos taurus*) have uncovered genetic diversity and historical patterns of gene flow not seen in analyses of the mtDNA control region (Cubric-Curik et al. 2022), and analyses of mitogenomes can provide additional resolution of regional structure (Shamblin et al. 2012). Future assessment of entire wolf mitogenomes and genome-wide profiles in this region and beyond might, therefore, uncover more variation and improve the resolution of spatial genetic structure.

The substructure of the Carpathians observed in this and previous studies could be influenced by the geomorphological division into three main sections: the Western, Eastern, and Southern Carpathians (Mráz and Ronikier 2016). This division largely coincided with our study and might have affected our results and the results of other studies including different taxa (e.g., Costolina alpina (*Hypochaeris uniflora*; Mráz et al. 2007), wild boar (*Sus scrofa*; Mihalik et al. 2020), the Eurasian lynx (*Lynx lynx*; Krojerová-Prokešová et al. 2019), and European mountain ash (*Sorbus aucuparia*; Yousefzadeh et al. 2021)).

Although primarily located within the Carpathian–Balkan cluster (C2), further study (hierarchical analyses) of population structure showed that wolves from the lowland Ukrainian steppe form a separate genetic group (C2A). Such division is in agreement with the results of previous studies (e.g., Pilot et al. 2006; Czarnomska et al. 2013; Stronen et al. 2013; Szewczyk et al. 2019). Our data also indicated that there have been some genetic connections through the Ukrainian steppe between wolves from the Carpathian and Caucasus regions based on the mtDNA haplotype distribution (w4, w7). We conclude that this region has played an important role in wolf gene flow and acts as a crossroad between western and eastern Eurasia.

The Dinaric cluster

The Dinaric wolves formed a separate cluster within the Dinaric–Balkan population, considered as one of the largest wolf populations in Europe (Hindrikson et al. 2016). At the European scale, wolves in the Dinaric–Balkan region are generally considered as one population (e.g., Kaczensky et al. 2013; Chapron et al. 2014), although recent analyses of mtDNA and microsatellite markers also show regional substructure between eastern (Balkan) and western (Dinaric) wolves (Djan et al. 2014; Šnjegota 2019; Šnjegota et al. 2021, this study). The Dinaric wolves showed substantially higher *Hd* and *B* values than the Balkan wolves, and a considerably lower number of haplotypes. Such results might indicate the increase in number of the Dinaric wolves after a strong bottleneck in the late 1980s (Boitani 2000; Kusak et al. 2000). The observed differentiation might also have been influenced by various other factors, including local adaptations (e.g., Pilot et al. 2006; Czarnomska et al. 2013; Kusak et al. 2018; Werhahn et al. 2018). This substructure requires further research using higher resolution molecular markers such as SNPs, and spatial data on landscape and environmental features.

The importance of the investigated regions in sustaining genetic diversity in wolves

The regions we investigated have been continuously inhabited by wolves, which allowed them to sustain considerable genetic diversity. The Carpathians and Balkans have emerged as reservoirs of historical genetic diversity, with modern genetic lineages overlapping and wolves dispersing toward neighboring populations (e.g., Hulva et al. 2018, this study). The Dinarics have served as a conduit for intra-population gene flow (Šnjegota et al. 2021) and contributed to recolonization of neighboring regions, including the Italian Alps (Fabbri et al. 2014; Ražen et al. 2016) and likely northward into regions such as Germany (Für Umwelt 2021) and Austria (Montana et al. 2017). The Alps have acted further

as the main corridor for dispersal of wolves from the Italian Peninsula toward France, Switzerland, Luxembourg, and Denmark (Fabbri et al. 2007; Boitani and Linnell 2015; Hindrikson et al. 2016; Schley et al. 2021). The Caucasus has emerged as a likely conduit for inter-continental gene flow, as it was shown to be connected with Western Asia and Eastern Europe (Pilot et al. 2014; this study).

The investigated regions have also provided suitable habitat and population sources for other large carnivores during varying environmental and anthropological circumstances, including brown bears (e.g., Chapron et al. 2014; Diserens et al. 2020) and Eurasian lynx (e.g., Breitenmoser et al. 1998; Palmero et al. 2021), and wild ungulates generally (Linnell et al. 2020).

Although the wolf is an ecologically flexible species capable of living in areas with considerable human presence, the investigated regions have been vital for preserving relatively large populations and, therefore, important genetic diversity for this and other wide-ranging species. Thus, these transboundary regions have an essential role in broad-scale planning toward conserving the evolutionary potential of wild species in a rapidly changing environment.

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Data availability The complete mtDNA sequences will be deposited in GenBank after the manuscript is accepted for publication.

Declarations

Conflict of interest The authors declare no competing interests.

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