Theriologia Ukrainica, **25**: 87–99 (2023)
p-ISSN 2616-7379 • e-ISSN 2617-1120
DOI: 10.53452/TU2508

GENETIC DIVERSITY OF mtDNA IN THE GREY WOLF POPULATION OF BELARUS THREATENED BY WOLF-DOG ADMIXTURE

Vladislav Molchan, Kanstantsin Homel, Arseni Valnisty, Mikhael Nikiforov, Ekaterina Kheidorova

Key words

wolf, dog, D-loop, mtDNA, hybridisation, genetic diversity, Belarus

doi

http://doi.org/10.53452/TU2508

Article info

submitted 21.05.2023
revised 22.06.2023
accepted 30.06.2023

Language

English, Ukrainian summary

Affiliations

Scientific and Practical Center for Bioresources, NAS of Belarus (Minsk, Belarus)

Correspondence
Kanstantsin Homel, Scientific and Practical Center for Bioresources, NAS of Belarus;
27 Akademičnaja Street,
Minsk, 220072 Belarus;
e-mail: homelkv@gmail.com;
orcid: 0000-0002-2396-1387

Abstract

Hybridisation with domestic species is one of the prominent threats to conservation of numerous valuable species in Belarus, in particular the grey wolf (*Canis lupus* Linnaeus, 1758), the population of which has previously shown unusually divergent genotypes, indicating a possible admixture with free-ranging dogs (*Canis lupus familiaris* Linnaeus, 1758). Such admixture could threaten the future of the species already weakened by anthropogenic pressure. Reports of hybrids in Belarus based on morphological characteristics have been steadily increasing in frequency since 2010, which can be tied with a growing food availability for feral dogs and low population density of grey wolf facilitating hybridisation. A limited number of genetic studies with sampling that partially covered Belarus did not detect any traces of hybridisation. Here we report our estimate of genetic diversity in the Belarusian population of grey wolf according to control region (D-loop) mtDNA sequence analysis. We analysed tissue samples from 35 specimens harvested during legal hunts across 25 administrative districts from all 6 regions of Belarus between 2009 and 2022, with 9 of the specimens reported to have morphological characteristics of wolf–dog hybrids. We detected 6 haplotypes among those 35 specimens, 4 of which were found among the likely wolf–dog hybrids. Clade analysis of the obtained sequences with 100 confirmed sequences of wolves, dogs, and their hybrids from the NCBI Genbank database has shown the presence of possible wolf–dog hybrids in our sample. The Belarusian wolf population has shown a low nucleotide and a relatively high haplotype diversity. The discovered genetic diversity data of the Belarusian wolf population is mostly in line with studies of similar wolf populations across Europe. The persistence of large wolf metapopulations across Eastern Europe and Russia, combined with the high mobility of the animal, seems to be the probable cause of genetic diversity of wolf populations in Belarus, but some degree of wolf–dog hybridisation could also explain the observed genetic heterogeneity, which invites further research based on nuclear markers.

Cite as

Molchan, V., K. Homel, A. Valnisty, M. Nikiforov, E. Kheidorova. 2023. Genetic diversity of mtDNA in the grey wolf population of Belarus threatened by wolf–dog admixture. *Theriologia Ukrainica*, **25**: 87–99. [In English]

Генетичне різноманіття популяції вовка у Білорусі за даними мтДНК в умовах гібридизації зі псами

Владислав Молчан, Костянтин Гомель, Арсеній Волністий, Михаіл Никифоров, Катерина Хейдорова

Резюме. Мета цього дослідження — оцінка генетичного розмаїття в популяції вовка (*Canis lupus* Linnaeus, 1758) на сучасному етапі та характеристика можливих гібридів зі псами (*Canis lupus familiaris* Linnaeus, 1758) у Білорусі на основі аналізу мінливості контрольного регіону (D-loop) мітохондріальної ДНК. Зразки біоматеріалу з білоруської популяції вовка, що використані в цьому дослідженні, отримані протягом 2009–2022 рр. Для проведення дослідження відібрано 35 зразків фрагментів м'язової тканини вовків, здобутих із 25 адміністративних районів, розташованих на території Брестської, Вітебської, Гомельської, Гродненської, Мінської та Могильовської областей Білорусі. При цьому 9 із 35 зразків отримані від особин, що мали виражені морфологічні ознаки вовко-псових гібридів. У порівняльному аналізі використано 100 нуклеотидних послідовностей контрольного регіону мтДНК вовків і псів, а також один зразок гібридної особини з бази даних нуклеотидних послідовностей NCBI. Загалом у вибірці вовка з Білорусі виявлено 6 гаплотипів контрольного регіону мтДНК. Характеризуючи рівень генетичної різноманітності білоруської популяції вовка, можна відзначити низьку нуклеотидну різноманітність і відносно високу гаплотипну різноманітність. Дев'ять особин, що можливо є вовко-псовими гібридами, представлені 4 гаплотипами з 6 виявлених для дослідженої вибірки. Отримані дані щодо генетичної різноманітності вовка в Білорусі узгоджуються з даними з наявних робіт зі схожої проблематики для європейських популяцій вовка. Високий ступінь мобільності вовка, збереження великих метапопуляцій виду в північно-східній Європі та в Росії всупереч високому ступеню антропогенного навантаження, а також певний рівень вовчої-псової гібридизації можуть виступати можливими причинами виявленої генетичної різнорідності у білоруській популяції вовка. Для отримання повнішої картини щодо генетичної різноманітності, так само, як і щодо ступеню і рівня гібридизації з безпритульними псами у білоруській популяції вовка необхідні подальші дослідження із залученням ядерних маркерів.

Ключові слова: вовк, собака, D-loop, мтДНК, гібридизація, генетичне різноманіття, Білорусь.

Introduction

The grey wolf (*Canis lupus* L., 1758) is one of the most widespread mammalian species in the world, with a range covering most of Eurasia and North America. Its numbers in Europe have significantly dwindled due to continuous human hunting efforts [Bibikov 1985], which also led to a diminished genetic diversity within populations, as well as a limit gene flow between them. In Belarus, the grey wolf is one of the most widespread large predators with significant presence across the country. Huge forested areas and low population density compared to Western and Central Europe create favourable conditions for wolf habitation. A 2013 study on the genetic structure of wolf populations across Europe reported the outcome of an SNP analysis of 177 wolves from 11 European countries, supplemented by mtDNA and microsatellite data [Stronen *et al.* 2013]. The analysis has shown anomalously high genetic diversity for the part of the sample originating from Belarus. As pointed out by the authors, the origin of this genetic diversity is unclear and requires further study [Stronen *et al.* 2013]. As no study into the genetics of Belarusian wolves has taken place since then, despite the species holding a crucial role in local ecosystems, we consider that this population requires more investigation into its genetic characteristics.

Among the significant threats to the conservation of the grey wolf in Europe is hybridisation with free-ranging dogs, as continuous gene introgression from dog can lead to a loss of wild variant genotype, endangering the long-term survival of the population [Gompert 2016]. While natural hybridisation can be associated with multiple positive evolutionary and conservational outcomes, such as genetic rescue [Brennan *et al.* 2014] or speciation [Lavrenchenko & Bulatova 2016], anthropogenic hybridisation is widely considered a threat to species conservation. Anthropogenic hybridisation is usually defined as hybridisation caused by deliberate or accidental human interference, such

as destruction of barriers between interfertile populations of different species, synanthropic invasions, introductions or migration directed by anthropogenic habitat loss, and can lead to loss of adaptational fitness in the emergent hybrid population [Rhymer & Simberloff 1996].

The issue of wolf–dog hybridisation threatening the fitness of wild wolf populations has been raised for many regions across Europe, including Portugal [Torres *et al.* 2017], Bulgaria [Moura *et al.* 2014], Latvia [Andersone *et al.* 2002], Italy and others [Randi *et al.* 2000]. Timely detection and estimation of hybridisation levels in wolf populations increasingly becomes a vital instrument for conservation and management of this canid predator. For this purpose, hybrid detection based on molecular genetic data, such as STR markers or full genome sequences are currently the most effective and reliable, as shown by such studies conducted in Italy [Dolf *et al.* 2000] and Fennoscandia [Smeds *et al.* 2021]. Among the recent studies on the subject it is vital to point out a 2022 inquiry into the genetics of the Italian wolf population by Lorenzini and colleagues [Lorenzini *et al.* 2022], in which the authors proposed a novel method for wolf–dog hybrid identification based on Bayesian analysis of 22 STR loci amplified in a multiplex PCR reaction. The method allows reliable hybrid identification, including classification of hybrids by generation, and we currently work on adopting this approach into research on wolf–dog hybridisation in Belarus.

Quantitative estimations of the degree and rate of wolf-dog hybridization in wild wolf populations are yet scarce. Such studies have been conducted in Bulgaria [Moura *et al.* 2014], Portugal [Torres *et al.* 2017], Latvia [Andersone *et al.* 2002], and the Iberian Peninsula [Ramirez *et al.* 2006; Godinho *et al.* 2011; Vilà & Wayne 1999], with results reporting hybrid presence with frequencies between 0% and 10.9% of the studied sample. In Belarus, the issue is particularly understudied. Probable cases of wolf–dog hybridisation based on morphological characteristics of animals have been since the 1980s, but limited genetic studies that partially covered Belarus did not detect any traces of hybridisation [Stronen *et al.* 2014]. In any case, reports of hybrids based on morphological characteristics have been steadily increasing in frequency since 2010, which can be tied with a growing food availability for feral dogs and low population density of the grey wolf facilitating hybridisation [Sidorovich & Rotenko 2019]. The situation invites a more thorough investigation of the hybridisation issue in Belarus.

In the present study, we conduct an estimation of genetic diversity of the Belarusian grey wolf population as well as a preliminary investigation into the possible signs of wolf–dog hybridization according to mtDNA control region (D-loop) sequence polymorphism.

Materials and Methods

Tissue samples used in the present study were harvested from 35 animals killed in legal hunts or traffic accidents between 2009 and 2022 (Supplementary material, Table S1). We used soft tissues harvested with sterile tools and stored in ethanol under cryogenic conditions. The sampling area included all six administrative regions (oblasts) and 25 administrative districts (rayons) (Fig. 1).

Additionally, nine of the samples were taken from specimens possessing atypical morphological characteristics, possibly indicating mixed wolf–dog ancestry (Supplementary material, Table S1, Fig. S1). No animals were harmed or killed specifically for the purpose of sampling.

For the clade analysis, we assembled a selection of 100 wolf mtDNA control region sequences for specimens harvested across Europe and adjacent regions, as well as one sequence of a confirmed wolf–dog hybrid (JN182126). The sequences were grouped into 8 regions—Eastern Europe, Northern Europe, Western Europe, Southern Europe, Eastern Asia, Central Asia, Western Asia, and the Caucasus—according to their place of origin (Fig. 2).

For DNA extraction, we used ‘Animal and Fungi DNA Preparation Kit’ (Jena Bioscience, Germany) following the manufacturer’s protocol. Obtained DNA isolates were measured for DNA concentration using NanoPhotometer P 330UV/Vis (IMPLEN, Germany) nanospectrophotometer and stored at -20°C for further use. We then amplified the control region’s HV1 fragment using primers L16462 (CATACTAACGTGGGGGTTAC) and H222 (AAACTATATGTCCTGAAACC) [Vilà *et al.* 1997] in standard 25 µl reactions with *Taq* polymerase using the following protocol: 1 cycle of

4 minutes at 94 ºC; 29 cycles of 30 seconds at 94 ºC, 45 seconds at 48 ºC, 1 minute at 72 ºC; and 1 final cycle of 5 minutes at 72 ºC.

Amplifications were carried out with a Bio-rad C1000 Touch (USA) thermo cycler. The obtained amplicons were examined for undesirable byproducts via gel electrophoresis in a 1.4 % agarose gel using a 15 cm MiniGel chamber (Bio-rad, USA) and Gel Doc™ XR+ gel documentation system (Bio-rad, USA). Sanger dideoxy sequencing of the amplified fragments was carried out using on base of the Institute of Bioorganic Chemistry of the NAS of Belarus. Amplicons were electrophoretically separated in 0.8% agarose gel, target fragments were cut out from the gel and purified with NucleoSpin Gel and PCR Clean-up (Macherey Nagel, Germany). Purified DNA fragments were then used in a sequencing reaction utilising BrilliantDye3.1 kit (Nimagen, Netherlands). Products were purified by ethanol/EDTA precipitation and separated with ABI3130 Genetic Analyzer (Thermo Fisher Scientific, USA). We then interpreted the obtained activity graphs into FASTA format sequences and aligned them with ClustalW algorithm using MEGA X v10.2.4. [Kumar *et al.* 2018].

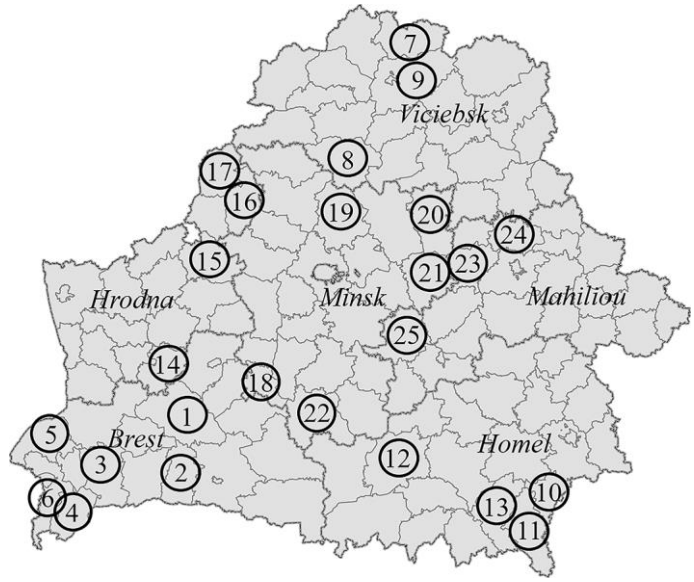

Fig. 1. Distribution of wolf sampling localities across administrative districts of Belarus. Black circles indicate that samples harvested in that district were used in the present study. Numbers within the circle correspond to location numbers Table S1 (column 'Location #').

Рис. 1. Розподіл місць збору вовка за адміністративними районами Білорусі. Чорні кола вказують на те, що зразки, зібрані в цьому районі, були використані в даному дослідженні. Числа всередині кола відповідають номерам пунктів у Таблиці S1 (колонка «Номер пункту»).

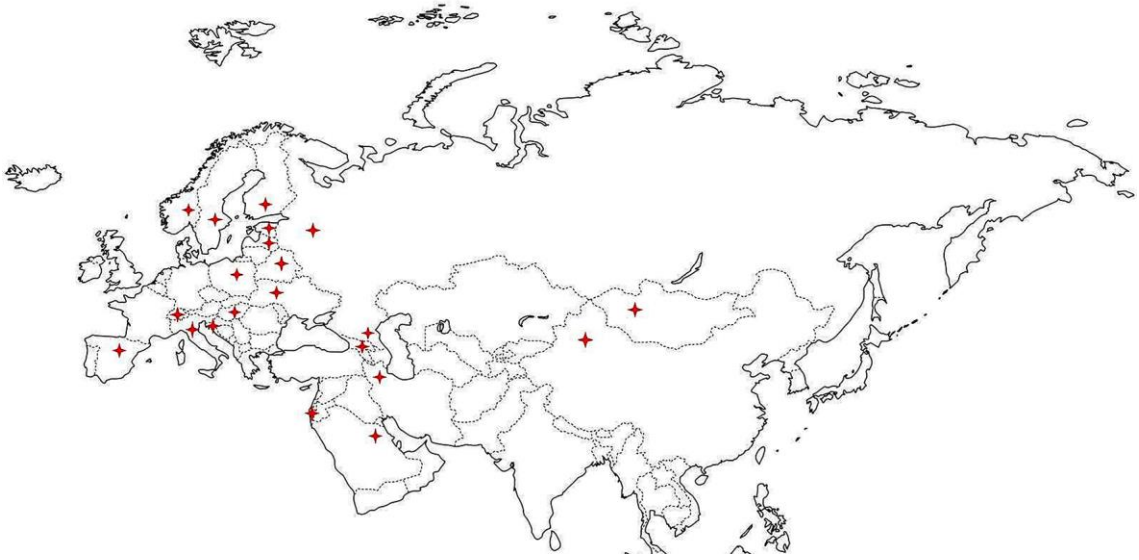

Fig. 2. Map showing locations from which wolf mtDNA D-loop region sequences from NCBI were taken.

Рис. 2. Карта, що показує місця, з яких були взяті послідовності області D-петлі мтДНК вовка з NCBI.

We used the same software to determine the number of polymorphic and parsimoniously informative sites in the obtained alignment. Estimation of genetic diversity indicators—nucleotide diversity (π), number of haplotypes (h), average number of nucleotide differences (k), and haplotype diversity (Hd)—was carried out in DnaSP ver. 6.12.03 [Rozas *et al.* 2017].

For the clade analysis, we aligned 100 wolf mtDNA control region sequences for specimens harvested across Europe and adjacent regions (Fig. 2) with the 35 sequences we obtained for the present study, and used this alignment to build a phylogenetic tree with MrBayes v3.2.7 [Huelsenbeck & Ronquist 2001]. We used the Hasegawa-Kishino-Yano model with Gamma distribution and invariant sites. Tree searches were conducted with 4 Markov chains (3 heated and 1 cold chain) with 2 million generations, sampling every 500 generations and with 25% of burn-in trees discarded. We then visualised this tree with FigTree v1.4.4[1]. We also built a haplotype network from the same alignment using median joining algorithm in Network 4.6.1.1 [Bandelt *et al.* 1999].

Fu's Fs, Tajima's D demographic tests were calculated in Arlequin ver. 3.5.2.2 [Excoffier & Lischer 2010], and Ramos-Onsin's and Roza's R_2 in DnaSP ver. 6.12.03 [Rozas *et al.* 2017]. Significant low values of R_2 and negative values of Fs and D indicate population expansion in the past [Ramos-Onsins & Rozas 2002]. P-values for R_2 was determined via coalescent simulation in DnaSP. Raggedness index (quantitative assessment of the smoothness of the mismatch distribution for the demographic scenarios of population expansion and stability in the past) and the sum of squared deviations (SSD) from the sudden expansion model [Rogers & Harpending 1992; Harpending 1994] were calculated in Arlequin by generating 10 000 simulated samples [Excoffier & Lischer 2010]. The abovementioned demographics tests included a sample of 32 wolf specimens, excluding the 3 most probable wolf–dog hybrids.

Results and Discussion

We managed to obtain control region sequence fragments for all 35 *C. lupus* specimens, with an aligned length of 302 base pairs. The alignment contained 9 polymorphic sites, 7 of which were parsimoniously informative. The sample presented six haplotypes. The nucleotide diversity index has shown a low value, while the haplotype diversity can be characterised as relatively high (Table 1). Table 2 presents a selection of estimates of genetic diversity in wolf populations from a number of similar studies.

As a main conclusion, the level of genetic diversity for the Belarusian wolf population is consistent with similar data from earlier studies of the wolf populations in Europe and can be characterised as moderately high.

Table 1. Genetic diversity and demographic history estimates for the Belarusian wolf population according mtDNA control region polymorphism

Таблиця 1. Оцінка генетичного різноманіття та демографічної історії білоруської популяції вовка за поліморфізмом контрольної ділянки мтДНК

Metric	Value	Metric	Value
N	35	Fu's Fs	2.497 NS
h	6	Tajima's D	1.59 NS
Hd ± SD	0.745±0.038	SSD	0.071 *
π ± SD	0.00977± 0.00075	Hri	0.163 NS
k	2.931	Ramos-Onsin's and Roza's, R_2	0.1893 NS

Note. N—sample size; h—number of haplotypes; k—average number of nucleotide differences; SD—standard deviation; Fu's Fs, Tajima's D, Ramos-Onsins and Rozas, R_2—population demography tests; SSD—sum of squared deviations from the sudden expansion model [Rogers & Harpending 1992]; Hri—Harpending's Raggedness index (r) [Harpending's 1994]; NS—statistically insignificant; *—$p < 0.05$.

[1] https://github.com/rambaut/figtree/releases

Table 2. A comparison of genetic diversity estimates for European wolf populations according to mtDNA control region polymorphism

Таблиця 2. Порівняння оцінок генетичного різноманіття європейських популяцій вовка за поліморфізмом контрольної ділянки мтДНК

Metric				Source
N	h	Hd ± SD	π ± SD	
947	27	0.88	0.022	[Pilot *et al.* 2010]
192	6	0.775 ± 0.014	0.020 ± 0.011	[Djan *et al.* 2014]
643	1–11	0–0.84	0.017 ± 0.009	[Pilot *et al.* 2006]
91	4	0.711 ± 0.018	0.018 ± 0.0096	[Gomerčić *et al.* 2010]
259	34	–	0.026 ± 0.014	[Vilà & Wayne 1999]
11	7	0.88	0.014	[Osman *et al.* 2016]
43 (Caucasus)	9	0.87	0.012	[Pilot *et al.* 2014]
74 (Bulgaria)	11	0.87	0.016	[Pilot *et al.* 2014]
7 (Spain)	2	0.29	0.004	[Pilot *et al.* 2014]

Note. N—sample size; h—number of haplotypes; Hd—haplotype diversity; π—nucleotide diversity; SD—standard deviation.

Analysis of the continental-scale Bayesian phylogenetic tree has revealed two main clades (Fig. 3). The first clade included mostly confirmed wolf specimens, with two dogs from Poland (GenBank: HM007199 and HM007196) and a confirmed wolf–dog hybrid from Estonia (JN182126), while the second clade was made up mostly from confirmed dogs, plus wolves from Mongolia (KU696392, KU696394, KU696395, and KU696396). All 35 Belarusian specimens obtained in this study ended up in the first clade, with mostly wolves, with 8 of those being specimens reported as likely hybrids due to morphological characteristics. One Belarusian specimen (CL87), also a morphologically likely hybrid, was assigned to clade 2. Two of our Belarusian specimens (CL54 and CL83) were also confirmed as wolf–hybrids by microsatellite analysis data (unpublished data). We see the distribution of Belarusian specimens across the tree as an indicator of high genetic heterogeneity of mtDNA lineages in the Belarusian grey wolf population, and a likely mixed origin of the population. The clade position of specimen CL87 also suggests the presence of rare wolf–dog hybridisation with matrilineal dog ancestry [Hindrikson *et al.* 2016].

Overall, the clade analysis seemingly indicates heterogeneous ancestry of the Belarusian wolves, as well as likely hybridisation. But due to inherent limitations of mtDNA data, we suggest that a more thorough inquiry utilising nuclear markers is required in order to resolve the issue of hybridisation in the studied population, as well as to model its genetic structure with sufficient accuracy and resolution for conservation purposes.

Our analysis of haplotype diversity shows the presence of 6 haplotypes among the 35 sampled specimens (Fig. 4, Supplementary material, Table S2), which largely agrees with earlier studies of mtDNA haplotype diversity in European wolf populations [Pilot *et al.* 2010]. The most common of those were haplotypes H1 (frequency of 37%), H6 (28%), and H8 (23%), while all the remaining haplotypes had occurrence rates between 3% and 6%. The three common haplotypes are encountered across most of Belarus, while the rare haplotypes H2 and H11 were only present in the south-eastern and south-western parts of the country, respectively, and haplotype H9 was detected in two localities. The limited sample size does not allow considering the presented variety and distribution of haplotypes complete for the studied region, but it does indicate a high degree of diversity and probably lack of structuring in the population of the species.

The alleged hybrids according to morphological characteristics belonged to four of the discovered haplotypes: H6, H8, H9, and H11 (Fig. 4). The distribution of the latter haplotypes is mainly tied to the central part of Belarus, but it hardly shows real presence of hybrid animals and rather reflects the data about possible wolf–dog hybrids based on information provided by hunters.

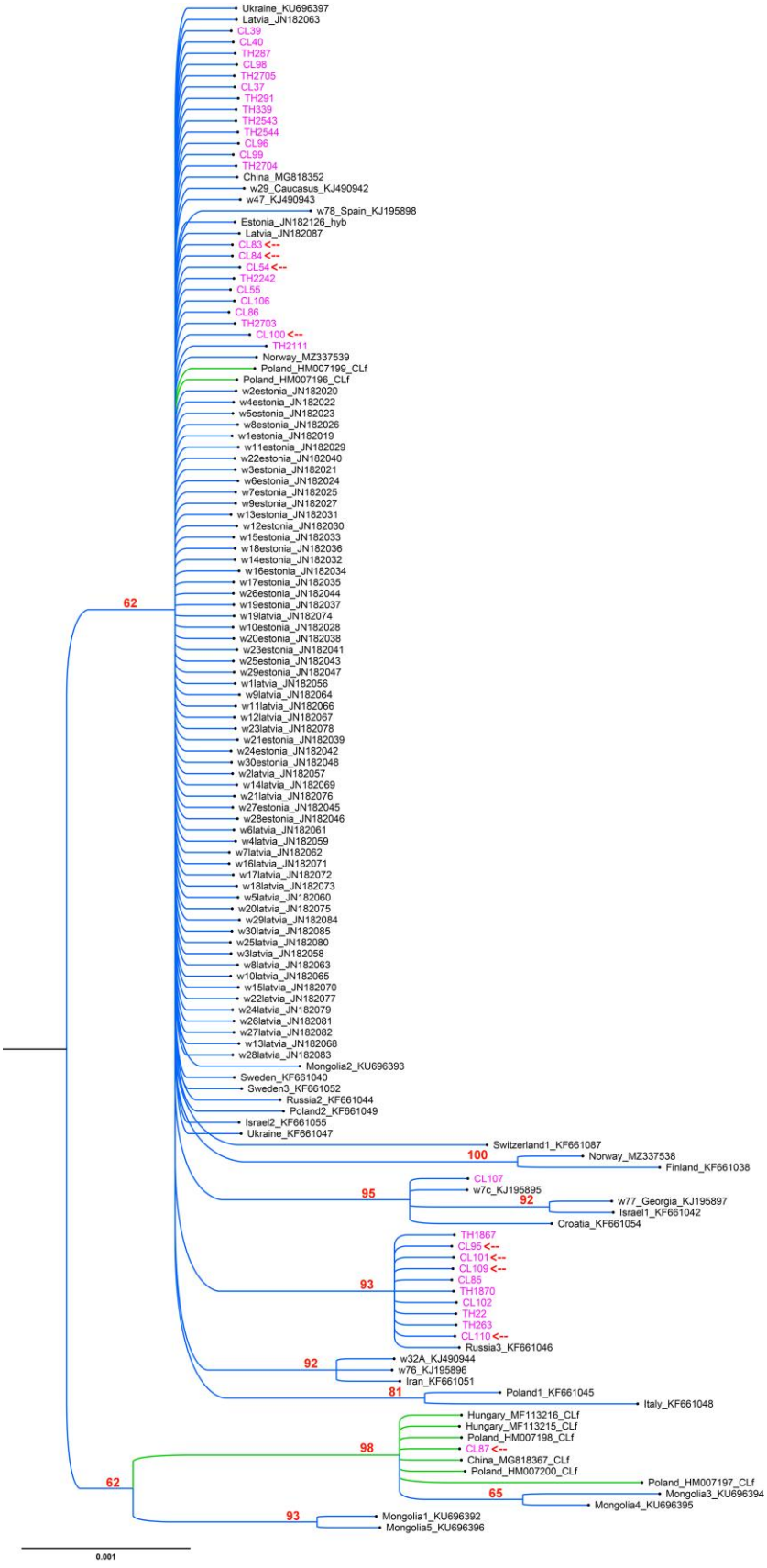

Fig. 3 (◄). Bayesian phylogenetic tree of wolves, dogs, and potential wolf–dog hybrids based on the analysis of mtDNA control region sequences.

Colours:

green—dogs, blue—wolves, red arrows—potential hybrids based on morphological features; purple labels—samples from this study.

Рис. 3 (◄). Байєсівське філогенетичне дерево вовків, псів та потенційних вовкопсових гібридів на основі аналізу послідовностей контрольних ділянок мтДНК.

Кольори:

зелений — пси, синій — вовки, червоні стрілки — потенційні гібриди за морфологічними ознаками, фіолетові мітки — зразки з цього дослідження.

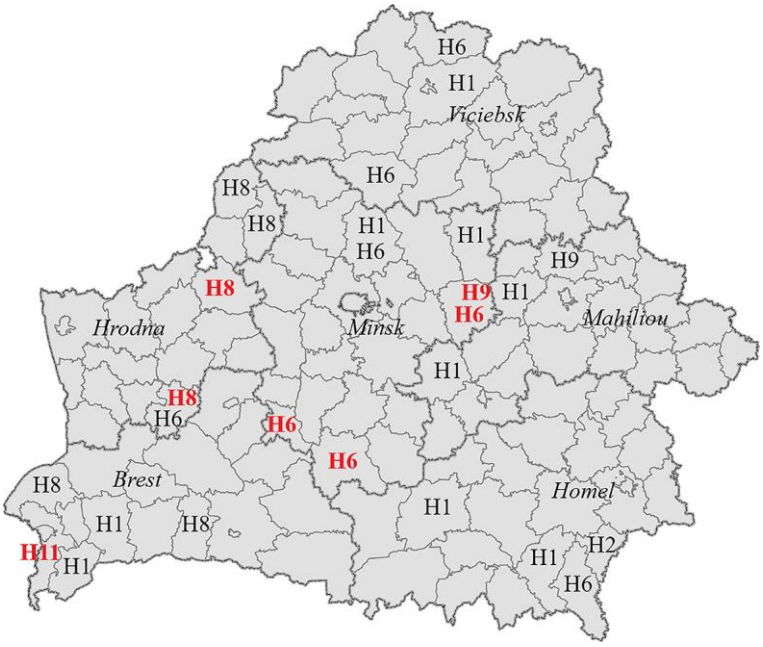

Fig. 4. Distribution of wolf and alleged hybrid haplotypes by sampling localities. Probable hybrids have their haplotypes given in bold red.

Рис. 4. Розподіл гаплотипів вовка та ймовірних гібридів за місцями збору. Гаплотипи ймовірних гібридів виділені жирним червоним кольором.

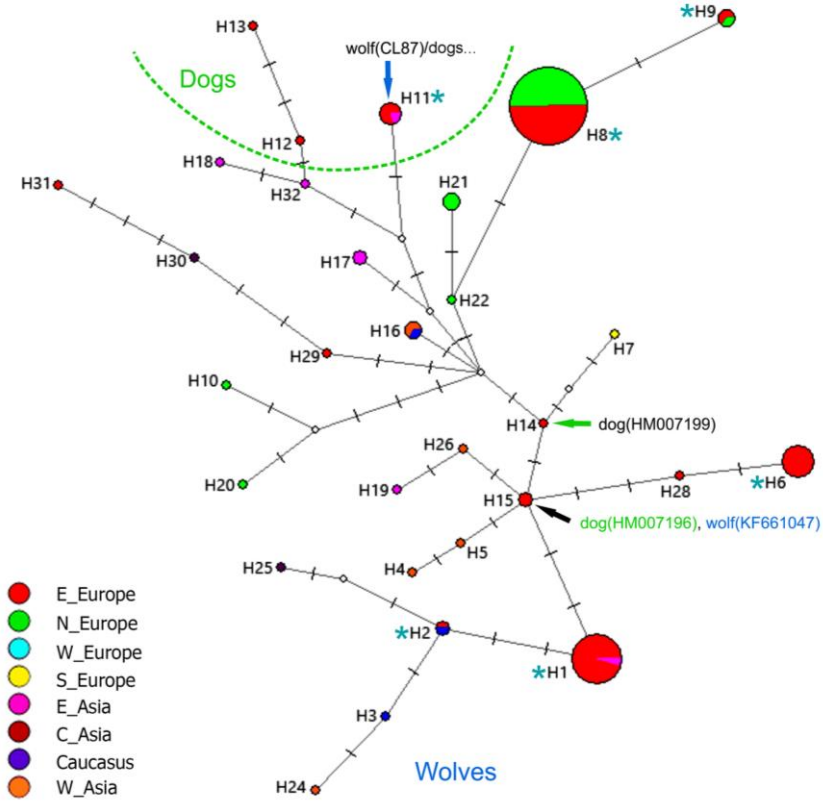

Fig. 5. A median joining network of haplotypes built from an alignment of 135 wolf and dog control region sequences.

Nodes correspond to haplotypes and are accompanied by haplotype names. Node size corresponds to the number of sequences assigned to the haplotype. Node colour denotes the geographic origin of samples assigned to the haplotype. Arrows indicate ambiguous haplotypes. Turquoise asterisk points out the haplotypes with samples from Belarus. Dashed green line isolates haplotypes found mostly in samples assigned to dogs.

Рис. 5. Медіанна мережа з'єднання гаплотипів, побудована на основі вирівнювання 135 послідовностей контрольних ділянок вовків і псів. Вузли відповідають гаплотипам і супроводжуються назвами гаплотипів. Розмір вузла відповідає кількості послідовностей, що відповідають гаплотипу. Колір вузла вказує на географічне походження зразків, віднесених до гаплотипу. Стрілками позначено неоднозначні гаплотипи. Бірюзовою зірочкою виділено гаплотипи зі зразками з Білорусі. Пунктирною зеленою лінією виділено гаплотипи, що зустрічаються переважно у зразках, які належать псам.

The analysis of the expanded alignment augmented by a continental selection of *C. lupus* mtDNA control region sequences obtained from NCBI Genbank database has shown a haplotype distribution similar to one shown in earlier studies of grey wolf phylogeography [Ersmark *et al* 2016]. We have observed 32 haplotypes in the alignment, with 19 of them being limited to Europe, 10 to Asia, and 3 being present in both regions (Fig. 5, Supplementary material, Table S2). Similar to the phylogenetic tree, the haplotype network created from the alignment does not indicate any clear ancestral structure for Belarusian grey wolves, or Eastern European wolves in general, as most haplotypes in question were spread across multiple regions. We interpret this as another indicator of mixed ancestry of the Belarusian wolf population.

Results of demographic tests for the Belarusian wolf population conclusively show the absence of drastic population growth events in the past, as indicated by non-significant test values of Fu's F, Tajima's D and R_2, and a significant deviation for the sudden expansion model (Table 1), agreeing with the structure of the obtained network of haplotypes.

We suggest that the modern Belarusian population of *C. lupus* originating in gradual admixture from multiple sources serves as the most likely explanation for the presented characteristics. Moreover, while large metapopulations of *C. lupus* inhabiting the neighbouring regions might be the main source of this admixture, our data does not exclude the possibility of feral dogs contributing a significant portion of contemporary grey wolf gene pool in Belarus. In fact, even the limited conclusions that can be drawn from mtDNA data seem to support the possibility of significant wolf–dog hybridisation occurring in Belarus. Further studies utilising nuclear markers would serve to decisively conclude on the degree of hybridisation in the population and on the severity of the issue. Increasing anthropogenic pressure on the wolf population and habitat fragmentation make dog admixture and its threat to the stability of the wolf population more probable with time. In this light, efficient management of the grey wolf in Eastern Europe is going to require a greater degree of genetic monitoring in order to retain the species' current degree of fitness and diversity.

Acknowledgements

The authors would like to thank all hunters who provided us with wolf samples, especially Professor Dr. Vadim Sidorovich for sharing with us his own wolf samples. We also thank the two anonymous referees for their important comments, Igor Zagorodniuk for editing and Zoltán Barkaszi for proofreading the article. The study was supported by the National Academy of Sciences of Belarus as a part of V. Molchan PhD dissertation 'Assessment of the genetic structure of the grey wolf (*Canis lupus*) population, occurrence and distribution of wolf-dog hybrids in Belarus'.

Author Contributions. Project administration and funding acquisition: M. Nikiforov, K. Homel, E. Kheidorova. Conceptualization and methodology: K. Homel, V. Molchan. Sample acquisition: M. Nikiforov, E. Kheidorova, V. Molchan. Sample processing and wetlab work: V. Molchan. Data analysis: V. Molchan, K. Homel, A. Valnisty. Writing: V. Molchan, K. Homel.

References

Andersone, Ž., V. Lucchini, J. Ozoliņš. 2002. Hybridisation between wolves and dogs in Latvia as documented using mitochondrial and microsatellite DNA markers. *Mammalian Biology*, **67**: 79–90. CrossRef

Bandelt, H. J., P. Forster, A. Rohl. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, **16**: 37–48. CrossRef

Bibikov, D.I. 1985. *The Wolf. History, Systematics, Morphology, Ecology.* Nauka, Moscow, 1–608. [In Russian]

Brennan, A. C., G. Woodward, O. Seehausen, V. Muñoz-Fuentes, C. Moritz, [*et al.*]. 2014. Hybridization due to changing species distributions: adding problems or solutions to conservation of biodiversity during global change*? Evolutionary Ecology Research*, **16**: 475–491.

Djan, M., V. Maletić, I. Trbojević, D. Popović, N. Veličković, [*et al.*]. 2014. Genetic diversity and structuring of the grey wolf population from the Central Balkans based on mitochondrial DNA variation. *Mammalian Biology*, **79**: 277–282. CrossRef

Dolf, G., J. Schläpfer, C. Gaillard, E. Randi, V. Lucchini, U. Breitenmoser, N. Stahlberger-Saitbekova. 2000. Differentiation of the Italian wolf and the domestic dog based on microsatellite analysis. *Genetics Selection Evolution*, **32**: 533–541. CrossRef

Ersmark, E., C. F. C. Klütsch, Y. L. Chan, M.-H. S. Sinding, S. R. Fain, [*et al.*]. 2016. From the Past to the Present: Wolf Phylogeography and Demographic History Based on the Mitochondrial Control Region. *Frontiers in Ecology and Evolution*, **4** (134): 1–10. CrossRef

Excoffier L., H. E. L. Lischer. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, **10**: 564–567.

Godinho, R., L. Llaneza, J. C. Blanco, S. Lopes, F. Álvares, [*et al.*]. 2011. Genetic evidence for multiple events of hybridization between wolves and domestic dogs in the Iberian Penin-

sula. *Molecular Ecology*, **20**: 5154–5166. CrossRef

Gomerčić T., M. Sindičić, A. Galov, H. Arbanasić, J. Kusak, [*et al.*]. 2010. High genetic variability of the grey wolf (Canis lupus l.) population from Croatia as revealed by mitochondrial DNA control region sequences. *Zoological Studies*, **49**: 816–823.

Gompert, Z., C. A. Buerkle. 2016. What, if anything, are hybrids: enduring truths and challenges associated with population structure and gene flow. *Evolutionary Applications*, **9**: 909–923. CrossRef

Harpending, H. C. 1994. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Hum. Biol*. **66**: 591-600.

Hindrikson, M., J. Remm, M. Pilot, R. Godinho, A. V. Stronen, [*et al.*]. 2017. Wolf population genetics in Europe: a systematic review, meta-analysis and suggestions for conservation and management: Wolf population genetics in Europe. *Biological Reviews*, **92**: 1601–1629. CrossRef

Huelsenbeck, J. P., F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17**: 754–755. CrossRef

Kumar, S., G. Stecher, M. Li, C. Knyaz, K. Tamura. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, **35**: 1547–1549. CrossRef

Lavrenchenko, L. A., N. Sh. Bulatova. 2016. The role of hybrid zones in speciation: A case study on chromosome races of the house mouse Mus domesticus and common shrew Sorex araneus. *Biology Bulletin Reviews*, **6**: 232–244. CrossRef

Lorenzini, R., L. Attili, C. Tancredi, R. Fanelli, L. Garofalo. 2022. A validated molecular protocol to differentiate pure wolves, dogs and wolf x dog hybrids through a panel of multiplexed canine STR markers. *Diversity*, **14**: 511. CrossRef

Moura, A. E., E. Tsingarska, M. J. Dąbrowski, S. D. Czarnomska, B. Jędrzejewska, M. Pilot. 2014. Unregulated hunting and genetic recovery from a severe population decline: the cautionary case of Bulgarian wolves. *Conservation Genetics*, **15**: 405–417. CrossRef

Osman İ., E. Aksöyek, S. Özcan, A. Keten, T. Yorulmaz, C. Tez. 2016. Genetic analysis of the Turkish gray wolf (Canis lupus) based on partial mitochondrial DNA sequences. *Vertebrate zoology*, **66**: 427–435.

Pilot, M., M. J. Dąbrowski, V. Hayrapetyan, E. G. Yavruyan, N. Kopaliani, [*et al.*]. 2014. Genetic variability of the grey wolf Canis lupus in the Caucasus in comparison with Europe and the Middle East: distinct or intermediary population? *PLoS ONE*, **9**: e93828. CrossRef

Pilot, M., W. Branicki, W. Jędrzejewski, J. Goszczyński, B. Jędrzejewska, [*et al.*]. 2010. Phylogeographic history of grey wolves in Europe. *BMC Evolutionary Biology*, **10**: article #100. CrossRef

Pilot, M., W. Jedrzejewski, W. Branicki, V. E. Sidorovich, B. Jedrzejewska, [*et al.*]. 2006. Ecological factors influence population genetic structure of European grey wolves. *Molecular Ecology*, **15**: 4533–4553. CrossRef

Ramirez, O., L. Altet, C. Enseñat, C. Vilà, A. Sanchez, A. Ruiz. 2006. Genetic assessment of the Iberian wolf Canis lupus signatus captive breeding program. *Conservation Genetics*, **7**: 861–878. CrossRef

Ramos-Onsins, S. E., J. Rozas. 2002. Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution*, **19**: 2092–2100.

Randi, E., V. Lucchini, M. F. Christensen, N. Mucci, S. M. Funk, G. Dolf, V. Loeschcke. 2000. Mitochondrial DNA variability in Italian and east European wolves: detecting the consequences of small population size and hybridization. *Conservation Biology*, **14**: 464–473. CrossRef

Rhymer, J. M., D. Simberloff. 1996. Extinction by hybridization and introgression. *Annual Review of Ecology and Systematics*, **27**: 83–109. CrossRef

Rogers, A. R., H. Harpending, 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, **9**: 552–569. CrossRef

Rozas, J., A. Ferrer-Mata, J. C. Sánchez-DelBarrio, S. Guirao-Rico, P. Librado, [*et al.*]. 2017. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. *Molecular Biology and Evolution*, **34**: 3299–3302. CrossRef

Sidorovich, V., I. Rotenko. 2019. *Reproduction biology in grey wolves Canis lupus in Belarus: Common beliefs versus reality*. Second edition, Chatyry chverci, Minsk, 1–223.

Smeds, L., J. Aspi, J. Berglund, I. Kojola, K. Tirronen, H. Ellegren. 2021. Whole-genome analyses provide no evidence for dog introgression in Fennoscandian wolf populations. *Evolutionary Applications*, **14**: 721–734. CrossRef

Stronen, A. V., B. Jędrzejewska, C. Pertoldi, D. Demontis, E. Randi, [*et al.*]. 2013. North-South Differentiation and a Region of High Diversity in European Wolves (*Canis lupus*). *PLoS ONE*, **8**: e76454. CrossRef

Torres, R. T., E. Ferreira, R. G. Rocha, C. Fonseca. 2017. Hybridization between wolf and domestic dog: first evidence from an endangered population in central Portugal. *Mammalian Biology*, **86**: 70–74. CrossRef

Vilà, C., P. Savolainen, J. E. Maldonado, I. R. Amorim, J. E. Rice, [*et al.*]. 1997. Multiple and Ancient Origins of the Domestic Dog. *Science*, **276**: 1687–1689. CrossRef

Vilà, C., R. K. Wayne. 1999. Hybridization between wolves and dogs. *Conservation Biology*, **13**: 195–198. CrossRef

Appendix: Supplementary material

CL83 CL84 CL109

CL110 CL95

Fig. S1. Photographs of presumably wolf specimens with morphological characteristics that suggest a possible dog ancestry (see relevant records in the Table S1).

Рис. S1. Фотографії імовірно вовчих особин з морфологічними ознаками, які вказують на можливе собаче походження (див. відповідні записи в таблиці S1).

Table S1. List of wolf and alleged wolf–dog hybrid specimens obtained in the present study

Таблиця S1. Перелік зразків вовка та ймовірних гібридів вовка і пса, отриманих у цьому дослідженні

Location #	Sample ID	Harvesting date	Harvesting location	Hybridisation clues
1	TH01867	03.04.2021	Bresckaja vobl., Ivacevicki rajon, v. Vyhanaščy	
2	TH02703	20.12.2021	Bresckaja vobl., Ivanaŭski rajon, v. Tyškavičy	
3	TH02704	28.12.2021	Bresckaja vobl., Kobrynski rajon, v. Haradziec	
4	TH02705	11.12.2021	Bresckaja vobl., Malarycki rajon, h. Malaryta	
5	TH02242	22.01.2018	NP Belaviežskaja pušča, Karaliova-Mastoŭskaje lias.	
6	**CL87**	March 2021	Bresckaja vobl., Brescki rajon	Oral suggestion from the hunter
7	TH01870	January 2021	Vitiebskaja vobl., Rassonski rajon, TC ‘Krasny Bor’	
8	TH00022	01.02.2016	Vitiebskaja vobl., Dokšycki rajon, h.p. Bjahomĺ	
9	CL37	2009	Vitiebskaja vobl., Polacki rajon	
9	CL39	2009	Vitiebskaja vobl., Polacki rajon	
9	CL40	2009	Vitiebskaja vobl., Polacki rajon	
10	CL107	03.04.2022	Homeĺskaja vobl., Lojeŭski rajon, v. Byvaĺki	
11	TH00263	20.01.2016	Homeĺskaja vobl., Brahinski rajon	
12	TH00287	winter 2016	Homeĺskaja vobl., Petrykaŭski rajon	
12	TH00291	winter 2016-2017	Homeĺskaja vobl., Petrykaŭski rajon	
13	TH00339	28.11.2016	Homeĺskaja vobl., Chojnicki rajon, Radzinskaje lias.	
14	**CL83**	26.04.2021	Hrodzenskaja vobl., Slonimski rajon, v. Azaryčy	Microsatellite data (unpublished data), morphological characteristics (Fig. S1)
14	**CL84**	26.04.2021	Hrodzenskaja vobl., Slonimski rajon, v. Azaryčy	Morphological characteristics (Fig. S1)
14	CL85	26.04.2021	Hrodzenskaja vobl., Slonimski rajon, v. Akačy	
15	**CL54**	2016	Hrodzenskaja vobl., Iŭieŭski rajon	Microsatellite data (unpublished data)
15	CL55	2016	Hrodzenskaja vobl., Iŭieŭski rajon	
16	CL86	2021	Hrodzenskaja vobl., Smargoński rajon	
17	CL106	25.11.2020	Hrodzenskaja vobl., Astravecki rajon, v. Belaja vada	
18	**CL95**	03.11.2021	Minskaja vobl., Kliecki rajon	Morphological characteristics (Fig. S1)
18	**CL109**	02.09.2022	Minskaja vobl., Kliecki rajon	Morphological characteristics (Fig. S1)
19	CL96	02.11.2021	Minskaja vobl., Lahojski rajon, v. Plieščanicy	
19	CL102	10.10.2020	Minskaja vobl., Lahojski rajon, v. Plieščanicy	
20	CL99	24.10.2021	Minskaja vobl., Krupski rajon, h. Krupki	
21	**CL100**	25.02.2021	Minskaja vobl., h. Bierazino	black wolf
21	**CL101**	15.02.2021	Minskaja vobl., h. Bierazino	light red coat colored wolf
22	**CL110**	08.12.2022	Minskaja vobl., Salihorski rajon, v. Rožan	Morphological characteristics (Fig. S1)
23	CL98	24.10.2021	Mahilioŭskaja vobl., Bialynicki rajon, v. Bialyničy	
24	TH02111	01.03.2021	Mahilioŭskaja vobl., Škloŭski rajon	
25	TH02543	December 2021	Mahilioŭskaja vobl., Asipovicki rajon, Asipovickaje lias.	
25	TH02544	December 2021	Mahilioŭskaja vobl., Asipovicki rajon, Asipovickaje lias.	

Note. Alleged hybrids have their Sample IDs given in **bold**; vobl.—region; v.—village; h.—town; h.p.—urban-type village; lias.—forestry.

Table S2. List of Canidae (wolves and dogs) mitochondrial DNA control region haplotypes according to a 302 bp alignment of 135 sequences including the Belarusian specimens obtained in the present study and 100 sequences obtained from the NCBI Genbank database

Таблиця S2. Список гаплотипів контрольної ділянки мітохондріальної ДНК Canidae (вовки, собаки) за вирівнюванням 302 п.н. 135 послідовностей, включаючи білоруські зразки, отримані в цьому дослідженні, та 100 послідовностей, отриманих з бази даних NCBI Genbank

Haplotype	Number of sequences	Sequences belonging to haplotype
H1	25	Ukraine KU696397, Latvia JN182063, CL39, CL40, TH287, CL98, TH2705, CL37, TH291, TH339, TH2543, TH2544, CL96, CL99, TH2704, China MG818352, w8latvia JN182063, w10latvia JN182065, w15latvia JN182070, w22latvia JN182077, w24latvia JN182079, w26latvia JN182081, w27latvia JN182082, w13latvia JN182068, w28latvia JN182083
H2	2	CL107, w7c KJ195895
H3	1	w77 Georgia KJ195897
H4	1	w29 Israel KJ490942
H5	1	w47 KJ490943
H6	12	TH1867, CL95, CL101, CL109, CL85, TH1870, CL102, TH22, TH263, CL110, Russia2 KF661044, Poland2 KF661049
H7	1	w78 Spain KJ195898
H8	61	Estonia JN182126 hyb, Latvia JN182087, CL83, CL84, CL54, TH2242, CL55, CL106, CL86, TH2703, w2estonia JN182020, w4estonia JN182022, w5estonia JN182023, w8estonia JN182026, w1estonia JN182019, w11estonia JN182029, w22estonia JN182040, w3estonia JN182021, w6estonia JN182024, w7estonia JN182025, w9estonia JN182027, w13estonia JN182031, w12estonia JN182030, w15estonia JN182033, w18estonia JN182036, w14estonia JN182032, w16estonia JN182034, w17estonia JN182035, w26estonia JN182044, w19estonia JN182037, w19latvia JN182074, w10estonia JN182028, w20estonia JN182038, w23estonia JN182041, w25estonia JN182043, w29estonia JN182047, w1latvia JN182056, w9latvia JN182064, w11latvia JN182066, w12latvia JN182067, w23latvia JN182078, w21estonia JN182039, w24estonia JN182042, w30estonia JN182048, w2latvia JN182057, w14latvia JN182069, w21latvia JN182076, w27estonia JN182045, w28estonia JN182046, w6latvia JN182061, w4latvia JN182059, w7latvia JN182062, w16latvia JN182071, w17latvia JN182072, w18latvia JN182073, w5latvia JN182060, w20latvia JN182075, w29latvia JN182084 w30latvia JN182085, w25latvia JN182080, w3latvia JN182058
H9	3	CL100, TH2111, Norway MZ337539
H10	1	Norway MZ337538
H11	5	Hungary MF113216 CLf, Hungary MF113215 CLf, Poland HM007198 CLf, CL87, China MG818367 CLf
H12	1	Poland HM007200 CLf
H13	1	Poland HM007197 CLf
H14	1	Poland HM007199 CLf
H15	2	Poland HM007196 CLf, Ukraine KF661047
H16	3	w32A KJ490944, w76 KJ195896, Iran KF661051
H17	2	Mongolia1 KU696392, Mongolia5 KU696396
H18	1	Mongolia3 KU696394
H19	1	Mongolia2 KU696393
H20	1	Finland KF661038
H21	1	Sweden KF661040
H22	1	Sweden3 KF661052
H24	1	Israel1 KF661042
H25	1	Croatia KF661054
H26	1	Israel2 KF661055
H28	1	Russia3 KF661046
H29	1	Poland1 KF661045
H30	1	Italy KF661048
H31	1	Switzerland1 KF661087
H32	1	Mongolia4 KU696395