



Wolf-dog hybrids in the eastern fringe Northern European wolf population on the background of increased hunting pressure

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Abstract

Recently in hunting pressure on the gray wolf (*Canis lupus lupus* L., 1758) population in Russian Karelia increased at an unprecedented rate. This fact also could suggest changes in the genetic structure of the population. The study region is of importance in the biogeographic sense—through this territory, the mammals populations of Scandinavia are connected to the Russian Plain, and further to Siberia. In this study, tissue samples of 35 wolves hunted in Karelia during 2012–2022 and 12 non-invasive specimens of dogs were used for genetic analysis. Four of the wolves (11%) were recognized by to phenotypic characteristics as wolf-dog hybrids or backcrosses. The analysis of autosomal microsatellites confirmed the admixed origin of three and excluded one. The mtDNA haplotype diversity was relatively low and represented by two haplotypes common for wolves in Eurasia and another one widely distributed in domestic dogs. The genetic diversity level of the population revealed by 14 microsatellite loci was high ($H_e = 0.746$; $H_o = 0.655$; $A = 8.1$). However, the observed heterozygosity proved to be notably lower than expected, and the inbreeding coefficient was also high ($F_{is} = 0.131$) and, moreover, higher than previously reported. We assume that the observed genetic processes, namely: interspecific hybridization and increased inbreeding occur against the background of an ongoing raise of wolf hunting in Karelia, which likely to, and became the cause of the observed processes.

Keywords *Canis lupus* L. · Population management · Mitochondrial and autosomal DNA · Microsatellites · Inbreeding · Introgression

Introduction

In most cases, an animal's phenotype is an important primary characteristic for identifying which its species. Most people are aware of the typical appearance of a gray wolf (*Canis lupus*); at least the usual characteristics found in northern Eurasia. There are color differences between wolves of the Old and New Worlds, and black morphs are extremely rare in Eurasia, as opposed to North America (Bibikov 1985; Yudin 2013; Apollonio et al. 2004; Pilot et al. 2019). It has been claimed that the black color variation in North American wolves is the result of an introgression of the dog's genome in the past; the genetic basis of inheriting this color variation has been studied quite closely, but it still seems to be under discussion (Anderson et al.

2009; Hedrick 2009; Cubaynes et al. 2022). We failed to find any mention in the literature of black-colored animals in Fennoscandia. In February 2021, one black wolf (GW_244) was shot legally in southwestern Russian Karelia (RK), near the Russian-Finnish border and a few km away from the Karelia-Leningrad Region border (61°19'47" N 29°26'10" E). A curious fact personally communicated by Ilpo Kojola is that there is a transboundary pack in Finland at the southern end of the Finnish-Russian border in which many, if not most, individuals are black.

Interspecific hybridization between wolves and dogs is a topical issue. Many studies across the world have examined various aspects and levels within this topic: local (Hindrikson et al. 2012; Molchan et al. 2023), worldwide or international (Pilot et al. 2019), legal status (Trouwborst 2014; Salvatori et al. 2020), hybrid identification (Kusak et al. 2018; Dziech 2021), proposals on unifying genetic identification approaches (Randi et al. 2014; Lorenzini et al. 2022), and many others. The level of knowledge of the population genetic structure varies notably across the wolf range

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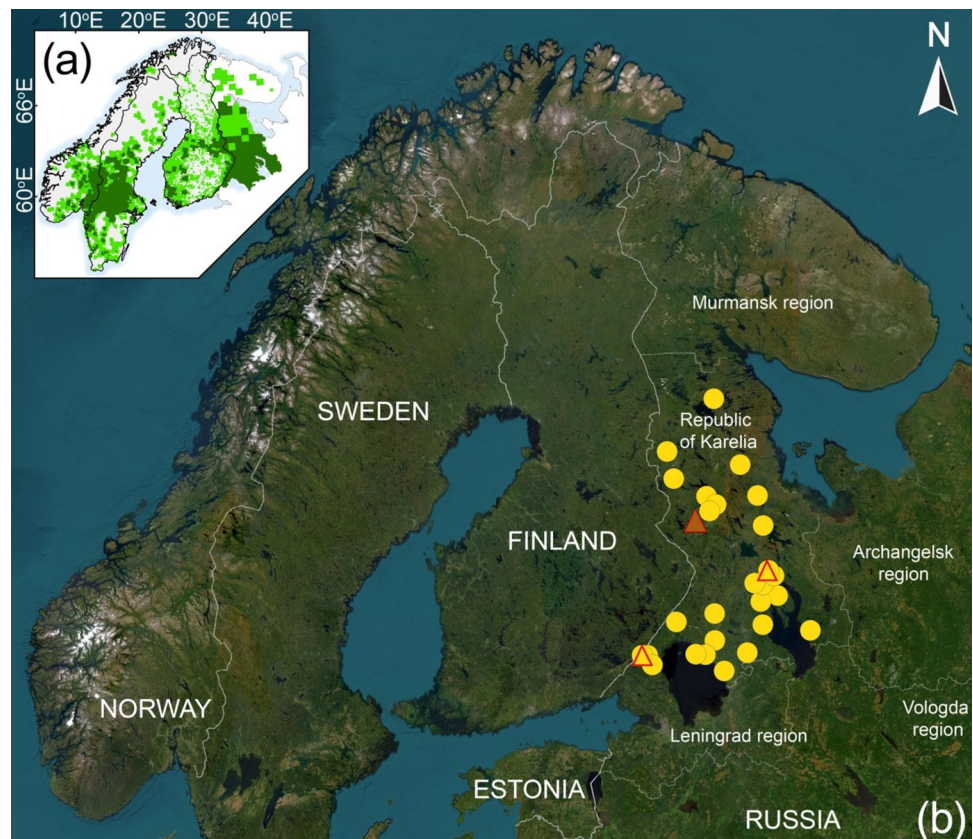
in Eurasia. Many European populations have been studied using genetic markers of various classes: mitochondrial DNA, autosomal and Y-linked microsatellites STR (Short Tandem Repeats), SNP (Single Nucleotide Polymorphism), and whole-genome analyses (Galaverni et al. 2017; Harmoinen et al. 2021; Hindrikson et al. 2016; Jansson et al. 2014; Pilot et al. 2007, 2018; Smeds et al. 2021; Sundqvist et al. 2001; Vilaça et al. 2023). At the same time, Russia's wolf range has been poorly studied and genetic issues of wolf-dog hybridization has been discussed in just a few studies (Pilot et al. 2019; Korablev et al. 2021; Kazimirov et al. 2022).

The focus in this study is determining the genetic status of wolves in Russian Karelia (RK). This population is important to ensure the connectivity between Fennoscandian wolves and the population of the East European Plain (Åkesson et al. 2022; Harmoinen et al. 2021). Studies of the wolf population in neighboring Finland, including some samples from RK, show the need for further genetic study of the wolf population in this region (Aspi et al. 2009; Jansson et al. 2012). Here we should mention that biogeographically, the "Karelian" and "Finnish" populations represent a single population without geographical barriers, which continues further to the east and south, and its division into two populations is rather nominal (Fig. 1) (Boitani 2003; Kojola et al. 2009). However, landscape and climatic conditions

and approaches to wolf management in Finland and Russian Karelia are completely different, which affects the dynamics of these population subunits (Suutarinen and Kojola 2017; Tirronen et al. 2023). Attempts have previously been made to assess the level of differentiation between these populations using different sets of autosomal STRs. Thus, Aspi et al. (2009) using 10, and Jansson et al. (2014) using 17 microsatellite markers found that the Finnish and Karelian populations were differentiated, whereas Åkesson et al. (2022) using a set of 26 autosomal STRs did not find significant differentiation between the populations.

There are no phenotype-based reports in the literature of hybrid individuals among RK wolves (Danilov et al. 1979; Danilov 2005). Microsatellite-based genetic studies found no wolf-dog hybrids (WDH) in 37 wolves covering the periods 1995–2000 and 2009–2010 either (Aspi et al. 2009; Jansson et al. 2012, 2014). A whole-genome study of Fennoscandian wolf populations did not detect admixed animals among 15 samples of RK wolves in the 2010s (Smeds et al. 2021). But, for the first time, an analysis of mtDNA in the sample of 90 RK wolves covering the period 2012–2022 detected an animal with a dog ancestry (Tirronen et al. 2023). However, there have been no targeted studies of the genetic structure and wolf-dog hybridization in the RK population. Urged by this information and the observations of wolves with phenotype deviations, we decided to study

Fig. 1 Wolves in Fennoscandia according to Kaczensky et al. (2021) supplemented by our data for the Murmansk region and Republic of Karelia **a**: dark green—permanent presence; light green—sporadic. Sampling locations in the Republic of Karelia **b**: circles—"pure" wolves, triangles—admixed individuals (yellow color is the "wolf" CR1 mtDNA haplotypes, brown—"dog" CR1 mtDNA haplotype)



possible cases of wolf-dog hybridization in RK. We genetically assessed whether there is evidence of mixing between wolves and dogs in Karelia using wild canids that seemed to be phenotypically deviated from wild wolves and a randomly assigned sample set.

Authors are also reasoning the consequences of the recent increase in hunting pressure for the wolf population, possibly manifested in interspecific hybridization, increased inbreeding, and changes in the genetic diversity and population structure.

Material and methods

Study area and wolf population

All samples were collected during 2012–2022 in Russian Karelia, which is situated in northwestern Russia (Fig. 1). It spans approximately 660 km north to south, and more than 420 km west to east, encompassing 172,400 km² (excluding the White Sea area) and more than half of the territory is boreal forest, almost a quarter is water bodies (rivers and lakes), a little less is wetlands, and the rest is farmland, settlements, and industrial areas.

A few remarks on the current status of the gray wolf in Karelia:

1. **Population size.** Since the beginning of the new millennium, the wolf population has ranged from about 280 to 450 individuals in February–March (the period of annual animal counts), i.e. at the end of the hunting season and before the offspring appear (Danilov 2005; Tirronen et al. 2023).
2. **Distribution.** Wolves live throughout RK, but the population is unevenly distributed—the population density in the north of the region (northern taiga subzone) is much lower than in the south (middle taiga subzone).
3. **Wolf hunting.** Since 2000, the annual harvest has varied from 45 to 236 animals. It did not exceed 100 before 2010, but since 2012 it has been steadily higher than 100, exceeded 160 in 2016 and reached an absolute peak of 236 individuals in 2018. In total, more than 2,700 wolves have been killed in Karelia since the beginning of the new millennium. A monetary bounty is paid to the hunter for each wolf they kill. Doubling of the bounties in 2016 increased the harvest. Since 2018, wolf hunters are additionally rewarded in the form of a license to hunt moose *Alces alces*, which has significantly stimulated wolf hunting. The reasons why the bounties are paid are the appearance of wolves in human settlements, attacks on dogs, and competition with man for wild ungulate resources, mainly moose.

4. **Phenotype.** Because bounties are paid, each killed animal undergoes a veterinary examination including evaluation of correspondence between the animal's body size and its approximate age and identification of morphological traits (color, head and tail shape, body proportions) corresponding to the biological species – the gray wolf.

Sampling and DNA extraction

In this study we used 47 canid samples, 36 of them belonging to wild canids and 11 to domestic animals (dogs). Among the 36 samples of animals killed in the wild, phenotypically 31 were wolves, 4 were hybrids, and 1 was a feral dog. For clarity, we should note that the terms “hybrid” or “WDH” we use also include backcrossed individuals. The set of 31 wolf samples was selected randomly to attempt to select samples as uniformly as possible in RK.

Before 2017, samples for genetic analysis accounted for 5–10% of the harvested individuals. Since then, the sampling rate was gradually raised to 85% of the annual wolf harvest. As hunters were to receive bounties for each wolf they killed, when there was uncertainty regarding animals with atypical wolf traits, their samples were sent to our laboratory for additional genetic studies. Thus, samples of four putative WDH were delivered to us.

All the wolves were killed legally and the samples were provided by the Hunting Department (RK Ministry of Natural Resources and Environment), which oversees hunting.

Muscle tissue or a piece of skin with muscle fibers were taken from the killed animals, placed in tubes with ethanol, and stored at – 20 °C until analysis.

To identify possible hybridization, 11 mongrel dogs were included in the analysis as a reference group. Samples from approximately “wolf-sized” dogs were obtained from a dog facility for stray or homeless dogs, which could, hypothetically, have participated in interspecific crossbreeding. Also, we used one sample of a feral dog (GW_50) trapped in the wild in southwestern RK. Thus, the total number of dog samples used in the analysis was 12. Dog hairs were put in paper bags and stored in a dark and cold place until analysis.

DNA was extracted from 20 to 50 mg of muscle or skin tissue or hair (5–10 root bulbs) using Qiagen DNeasy Tissue kit (Qiagen) following the manufacturer's instructions.

All sampling locations, mtDNA haplotypes and the genetic status of animals based on autosomal microsatellites were entered in an Excel spreadsheet. Based on this data, a GIS of the study area incorporating the analyzed data was created using QGIS 3.4.14.

Microsatellite analysis and sex identification

To obtain individual genetic profiles of wolves, we chose a set of 20 microsatellite markers: CPH2, CPH4, CPH5, CPH8, CPH12 (Fredholm and Wintero 1995), FH2001, FH2017, FH2010, FH2054, FH2079, FH2088, FH2096 (Francisco et al. 1996), AHT130, AHT137 (Holmes et al. 1995), INRA21 (Mariat et al. 1996), AHTk211 (Lingaas et al. 1997), vWF (Shibuya et al. 1994), and C09.173, CXX279, C20.253 (Ostrander et al. 1995). The forward primer for each locus was labeled with fluorescent dye: FAM, ROX, TAMRA, R6G. All loci were polymerase chain reaction (PCR) amplified in a volume of 25 µl containing 16.5 µL H₂O, 5 µL Screen Mix (Evrogen), 1 µL of each primer (10 µM, Syntol), and 1.5 µL of matrix DNA. PCR protocol: initial denaturation at 95 °C for 3 min, 35 cycles, including 20 s at 95 °C, 20 s at 58–62 °C, and 20 s at 72 °C, and final elongation for 5 min at 72 °C.

To identify the length of the amplified loci, 0.5 µl of the molecular size standard GeneScan™ 600 LIZ (Applied Biosystems, USA) was added to PCR products. The fragment length was determined using capillary electrophoresis on a Seqstudio genetic analyzer (Applied Biosystems, USA) following the protocol provided by the manufacturer. The results were analyzed in GENEMAPPER v. 4.0 (Applied Biosystems, USA). Some samples were repeated twice to avoid genotyping errors. The presence of null alleles and stuttering were analyzed with MICRO-CHECKER v2.2. (van Oosterhout et al. 2004). Sex identification of putative hybrids was performed as described in Sastre et al. (2009).

Control region I mtDNA sequencing

Partial sequences (350 bp) of the hypervariable part of the mitochondrial DNA control-region I (CR1mtDNA) (Saccone et al. 1987) were obtained using universal PCR primers Thr-L 15926 5'-CAATCCCCGGTCTTGTAAC-3' and DL-H 16340 5'-CCTGAAGTAGGAACCAGATG-3' (Vilà et al. 1999) from wild animals. The amplification of mtDNA control region I fragments was carried out the same way as in microsatellite analysis at 52 °C annealing temperature. The nucleotide sequences of the amplified mtDNA region were determined according to the Sanger method in two directions using BigDye Terminator 3.1 DNA sequencing kits (Applied Biosystems, United States) on Seqstudio genetic analyzer (Applied Biosystems). The obtained sequences were edited manually and aligned in MEGA11 (Tamura et al. 2021) using a ClustalW algorithm. The haplotype (Hd) and nucleotide (Pi) diversity were calculated using the DnaSP 5.0 software (Librado and Rozas 2009).

Genetic diversity

GENALEX software (Peakall and Smouse 2012) was used to calculate the number of alleles per locus (A), observed heterozygosity (Ho), expected heterozygosity (He), and the test for the Hardy–Weinberg equilibrium (HWE) in wolves separately. The inbreeding coefficient (Fis) and its statistical significance were calculated using ARLEQUIN v.3.5 (Excoffier and Lischer 2010).

To identify a possible bottleneck signature in the Karelian wolf population, we used the BOTTLENECK v1.2.02 software (Cornuet and Luikart 1996). The TPM mutation model was chosen as the most appropriate for microsatellite data and a probability of 95% for single-step mutations with a variance of 12 was applied, as suggested by S. Piry et al. (1999). We used the Wilcoxon's test to determine if a significant excess of heterozygosity was detected (Luikart and Cornuet 1997). Also, a mode shift test was applied to determine if the frequency classes based on allele size deviated from the normal L-shaped distribution, indicating rare allele loss.

Analysis of wolf-dog hybridization

To identify wolf-dog hybrids (WDH) and admixed individuals, we used genotypes from both the wild wolf population and dogs. STRUCTURE v2.3.4 (Pritchard et al. 2000) was applied to evaluate the number of genetic clusters (K) in the data and to assign individuals to their likely origin. The dataset for identifying hybrid samples comprised 47 individuals genotyped using 14 microsatellite loci (STR), including wolves ($n=31$), dogs ($n=12$), and four putative hybrids by phenotype. The run parameters were the following: “admixture” and independent allele frequency “I” models, without any prior population information, assuming K from 1 to 10. Five independent runs were made for each K using 1,000,000 sweeps of the Monte Carlo Markov chain (MCMC) and discarding the first 100,000 as burn-ins. The results were then processed in STRUCTURE HARVESTER (Earl and vonHoldt 2012). The ΔK statistics were used to identify the highest rate of increase in the posterior probability LnP(D) of the data between each consecutive K, as described by Evanno et al. (2005). CLUMPP (Jakobsson and Rosenberg 2007) was used to match the data from the multiple runs for each K and STRUCTURE Plot (Ramasamy et al. 2014) was used to display the results. The threshold level for differentiating wolves and dogs from admixed individuals was determined based on the analysis of simulated individuals with previously known ancestry. We used reference wolf ($q_{\text{wolf}} \geq 0.98$) and dog ($q_{\text{dog}} \geq 0.98$) samples to simulate 30 genotypes for each of the 6 ancestry classes (pure wolves, pure dogs, F1 and F2 hybrids, and first-generation backcrosses) in HYBRIDLAB 1.0 (Nielsen et al. 2006). The

simulated genotypes were analyzed in STRUCTURE 2.3.4 with the same parameters mentioned above.

Separate analyses to distinguish between wolves, dogs, and wolf-dog hybrids on the basis of microsatellite data with factorial correspondence analysis (FCA) were done in GENETIX v. 4.05 (Belkhir et al. 2004).

To test if these wolves were related, we calculated the maximum likelihood of relatedness using ML-RELATE program (Kalinowski et al. 2006).

NEWHYBRIDS v1.1 beta (Anderson and Thompson 2002) was used to calculate the posterior probability on MCMC estimates that the individual belongs to each of the 6 predefined ancestry classes: wolf, dog, hybrids F1 and F2, and respective first-generation backcrosses to wolf (BxW) and dog (BxD). The overall data set was processed with 1,000,000 MCMC sweeps.

Results

Phenotype

According to the veterinary expertise based on phenotype, of the entire set of samples ($n=35$), four individuals were preliminarily assigned to the WDH category. The traits by which these individuals are distinguished from the wolf general set were variable among individuals. GW_119 was an animal of close to synanthropic way of living at the outskirts of a city with a population of about 14,000 people. The distinctive traits of GW_119 were white claws and white pads. GW_244 was a completely black wolf-like animal (Fig. 2) that lived in a pack of typical wolves and was shot together with an animal with a typical wolf phenotype from the same pack. Sample GW_246 was not supplied with an exact description, but was accompanied by a note “hybrid”. The adult female GW_494 had no black stripes on its front legs, no black tip of the tail, the overall color tone was brownish red, and the shape of the head resembled the Akita Inu breed (Fig. 2). These four WDHs were killed during the period 2017–2022, during which 1127 wolves were harvested. Thus, the share of the presumed phenotypic hybrids was 0.35%.

CR1 mtDNA

Sequences of the mtDNA control region were obtained for 36 individuals taken in the wild population (35 wolf-like and 1 feral dog). The analysis revealed three haplotypes, 2 of wolves and 1 of dogs, the number of polymorphic sites was 8, haplotype diversity $H_d=0.541 \pm 0.034$, and nucleotide diversity $P=0.00854 \pm 0.00060$. Among the wolves in the sample, 18 wolves had the haplotype with GenBank accession number OP503597 (hereafter H1) and 16 had the

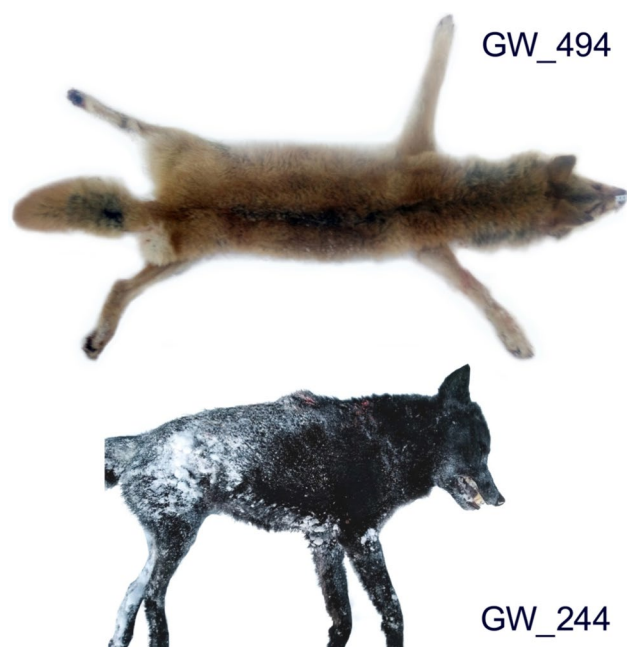


Fig. 2 The skin of the putative wolf-dog hybrid GW_494, genetically recognized as “purebred” wolf, and the general appearance of the wolf-dog backcross GW_244 (photos provided by local hunting authorities)

haplotype OP503598 (H2), both were previously known to be common in RK (Tirronen et al. 2023). Two samples (GW_246 marked as a “hybrid” and the feral dog GW_50) had a haplotype (H3), was identical to the homologous CR1 mtDNA sequence widely distributed in domestic dogs (Desmyter and Gijssbers 2012; Hindrikson et al. 2012; Marinov et al. 2018, etc.) and rare in wolves (Pilot et al. 2010; Ersmark et al. 2016). The nucleotide sequences were uploaded to GenBank with the accession numbers PQ213603 for GW_50 and PQ213604 for GW_246. Information about the haplotypes of putative and identified admixed individuals is presented in Table 1.

Genotyping errors

All the samples were genotyped using 20 microsatellite markers, but we were only able to obtain reliable allele length for 14 loci (AHT137, CPH2, CPH8, CPH4, CPH12, FH2010, FH2096, AHTk211, vWF, FH2001, CXX279, C09.173, CPH5, FH2054). Six loci were excluded from further analysis due to the appearance of false alleles. The MICRO-CHECKER program analyzed 35 genetic profiles for genotyping errors and identified 5 loci (FH2096, CXX279, C09.173, CPH5, FH2054) with heterozygosity deficiency suggesting the presence of null alleles in these loci. However, given the regular and intense persecution of wolves in the studied population, we expected an increase

Table 1 Identifications of membership proportion of wolves by STRUCTURE and ancestry classes by NEWHYBRIDS for phenotypic hybrids found in the wolf population of Russian Karelia

Sample	Year	Location	CR1 mtDNA haplotype	Phenotypic trait	Sex	STRUCTURE: q_wolf (90% BCI)	NEWHYBRIDS: ancestry proportions					
							Wolf	Dog	F1	F2	BxW	BxD
GW_119	2018	62°55'44" N 34°28'55" E	H1	White claws, white pads	f	0.8300 (0.484–1.000)	0.03332	0.00000	0.00017	0.16274	0.80376	0.00001
GW_244	2021	61°19'47" N 29°26'10" E	H2	Completely black color	m	0.8314 (0.198–1.000)	0.56955	0.00000	0.00002	0.11917	0.31111	0.00014
GW_246	2021	63°47'N 31°39'E	H3	Marked as a “WDH” (without details)	m	0.2720 (0.000–0.726)	0.00009	0.00002	0.00737	0.71626	0.18097	0.09529
GW_494	2022	64°16'30"N 34°05'10"E	H1	No black tip on tail and black stripes on front legs	f	0.9838 (0.890–1.000)	0.96416	0.00000	0.00000	0.00593	0.02991	0.00000

Table 2 Genetic diversity for 14 microsatellite loci in the Russian Karelia wolves ($n=35$) including admixed individuals: expected (He) and observed (Ho) heterozygosities, number of alleles (A), and inbreeding coefficients (Fis)

Locus	He	Ho	A	Fis
AHT137	0.872	0.828	10	0.068
CPH2	0.744	0.633	8	0.165
CPH8	0.763	0.655	8	0.158
CPH4	0.794	0.667	6	0.177*
CPH12	0.632	0.700	7	– 0.091
FH2010	0.701	0.633	5	0.113
FH2096	0.760	0.690	5	0.110
AHTk211	0.441	0.464	4	– 0.035
vWF	0.769	0.700	7	0.107
FH2001	0.711	0.733	5	– 0.015
CXX279	0.865	0.667	14	0.245*
C09.173	0.802	0.633	11	0.226*
CPH5	0.814	0.567	13	0.319*
FH2054	0.776	0.600	10	0.246*
Mean	0.746	0.655	8.1	0.131
SE	0.029	0.022	0.835	

* $p < 0.05$

in the proportion of closely related crossbreeding between individuals. As we show later, the level of inbreeding in the population has increased, resulting in deviations in the Hardy–Weinberg equation. Because these primers were developed for the wolf and tested multiple times on different populations, all 14 loci were used to assess the genetic diversity of the wolf population, its demographic history, and interspecific hybridization.

Genetic diversity of the wolf population

All 14 microsatellite loci were polymorphic and the diversity was relatively high: $Ho=0.655$ ($SE=0.022$), $He=0.746$ ($SE=0.029$), $A=8.1$ ($SE=0.835$) (Table 2). Four loci (CPH2, CXX279, CPH5, FH2054) were significantly out of HWE ($P \leq 0.005$) in the pooled data set of 35 samples. The overall inbreeding coefficient in the studied population was positive and differed significantly from zero ($Fis=0.131$, $P=0.0000$). Also, the inbreeding coefficient was significantly positive in five loci (CPH4, CXX279, C09.173, CPH5, FH2054). These genetic diversity parameters show the results for 35 individuals, i.e. including admixed individuals, because, in our opinion, they are also part of the wild wolf population. However, to evaluate contribution of admixed individuals to the genetic diversity of the study sample, we provide average data for 31 individuals (without putative WDH): $Ho=0.662$ ($SE=0.023$), $He=0.751$ ($SE=0.028$), $A=8.2$ ($SE=0.833$), $Fis=0.136$ ($P=0.0000$).

Population bottleneck

The Wilcoxon's test for heterozygosity excess was performed in two repeats using 14 microsatellite markers for whole sample ($n=35$) and excluding three wolf-dog admixed individuals. It suggested no bottlenecks in either case ($P=0.932$ and $P=0.879$). Also, the allelic frequency distribution for the Karelian population was L-shaped in both cases, as expected for populations that have not experienced recent bottlenecks.

Genetic identification of WDHs

Clustering using Bayesian approaches in STRUCTURE software was performed on the entire data set for 14 loci with the number of inferred clusters increasing from $K=1$ – $K=10$. ΔK analysis showed a high peak when $K=2$. The genetic profiles were clearly divided according to their ancestry into the wolf and dog groups with the average $q_{\text{wolf}}=0.982$ ($n=32$) (0.910–0.997, 90% BCI 0.551–1.000) and $q_{\text{dog}}=0.990$ ($n=12$) (0.969–0.997, 90% BCI 0.767–1.000). Bayesian approaches in assessment of simulated genotypes of different ancestry classes revealed the average threshold $q_{\text{wolf}}=0.953$ (0.896–0.973, 90% BCI 0.699–1.000) and similar for dogs $q=0.952$ (0.879–0.973, 90% BCI 0.644–1.000). As the analyzed population is actively harvested, there may be an influx from outside, in which case immigrants may be mistakenly recognized as admixed individuals (Harmoinen et al. 2021). For this reason, and to avoid the Type I error i.e., erroneously identify a wolf as a hybrid (Dziech 2021), a relatively high threshold of $q_{\text{wolf}}=0.900$ was established to

distinguish “pure” wolves from admixed animals. It should be noted that in the context of our study, by “pure” we mean wolves defined without a reasonable doubt as “purebred” i.e. “unadmixed” by the used set of microsatellite markers and established q threshold. Using $q_{\text{wolf}}=0.900$, we obtained 97% purebred wolves among the simulated ones, 100% of simulated F1 and F2 hybrids, were assigned to introgressed individuals, and 90% of first-generation backcrosses to wolf also were identified as introgressed. At the same time, in the analyzed sample ($n=35$), 91% of wolves had q values >0.900 and were classified as conditionally “purebred”.

Three individuals appeared to be of mixed ancestry, with q_{wolf} values of 0.272 (GW_246), 0.830 (GW_119), and 0.831 (GW_244), and they were therefore identified as WDH. They accounted for 8.6% of the wolf sample. Several individuals had parameters close to the threshold value for q_{wolf} , so they are also shown in Table 1 and in Fig. 3.

The results of the NEWHYBRIDS analysis (Fig. 3, Table 1) generally confirmed the results of the STRUCTURE analysis. There was a clear partition of the sample into wolves and dogs, and individuals of admixed origin were also identified. As for admixed individuals, GW_246 was determined to be an F2 (72%), the other two samples (GW_244, GW_119) were assigned to first-generation backcrosses to wolf (31 and 80%, correspondingly).

In the FCA analysis, wolves and dogs were clearly distinguished from each other based on the distributions of allele frequencies at 14 microsatellite loci (Fig. 4). Positions of the samples with mixed ancestry were different. Only the GW_246 sample clearly departed from the “pure” wolf cloud. In a previous analysis it was detected as a F2. Two

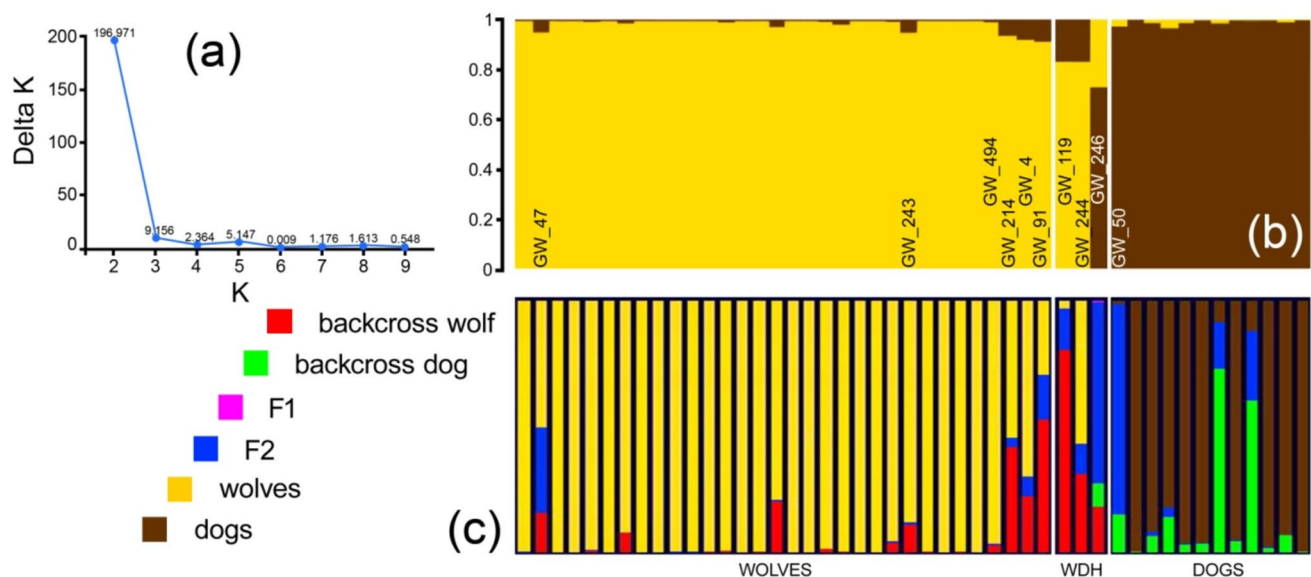
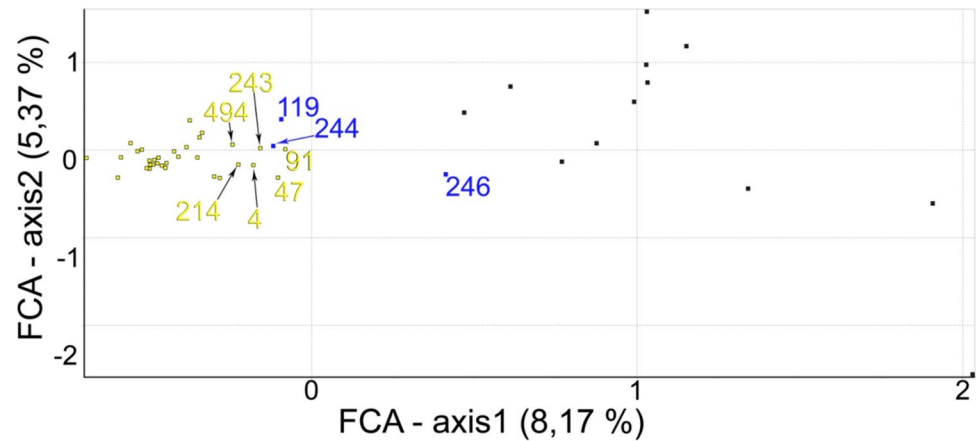


Fig. 3 Genetic differentiation of wolves, dog, and admixed individuals: **a** K values (Evanno et al. 2005); bar plotting outputs of the analysis from STRUCTURE **b** and NEWHYBRIDS **c**

Fig. 4 FCA by 14 STRs illustrating the genetic distribution of dogs (black dots), wolves (yellow squares), and admixed individuals (blue dots)



individuals of mixed origin, GW_244 and GW_119, were placed at the fringe of the wolf cloud, tending towards the dogs. The black wolf GW_244, identified as an admixed individual, was taken together with GW_243 from the same pack. It was established that these wolves were not related. The maximum likelihood estimate of relatedness was 0.0030.

Of the four individuals that had phenotypic deviations from the wolf normal appearance or were marked as a “hybrid”, in two cases (GW_119, GW_244) the hybrid status was supported only by STR, and in one case (GW_246) by STR and mtDNA analyses. The GW_494 individual with a dog phenotype was surprisingly recognized as a wolf by both marker classes (Table 1, Fig. 3). For this sample (GW_494), we repeated all stages of analysis since DNA extraction three times. Genetic analysis of 14 microsatellite markers did not reveal additional clearly admixed individuals than those phenotypically detected, but excluded an individual with obvious phenotypic deviations from the “wolf” norm (GW_494) from the number of WDHs.

Discussion

During the period from 2017 to 2022, four wolves (GW_119, GW_244, GW_246, GW_494) with phenotypic deviations suggesting WDH were removed from the Karelian wolf population. In three cases (GW_119, GW_244, GW_246) this determination was confirmed genetically. The dog mtDNA haplotype in GW_246 indicates that this WDH appeared as a result of a crossing between female dog and male wolf. Such an event of interspecific crossing is interesting and rare. There is a typical sexual disproportion in the hybridization of wolves and dogs in the wild, where a lone female wolf chooses a male dog as a pair (Hendrickson et al. 2012). The GW_246 sample comes from the northern taiga subzone of Karelia, where the wolf population density is relatively low. Perhaps the combination of a sparse population and

intensive human persecution were sufficient for a rare hybrid type (more likely F2) to appear (Table 1, Fig. 3). Another hybrid of unusual appearance—GW_244, was strikingly different from the average Karelian wolf in having a totally black coat. The common color of Karelian wolves is a variety of shades of light and dark gray, often in combination with reddish color, generating a color variation from almost straw to dark gray, but black wolves have never been found there (Danilov 2005). It is known that black wolves are common in some populations of North America, but the emergence of such individuals in European populations is extremely rare event associated with possible hybridization with dogs (Gipson et al. 2002; Musiani et al. 2007; Caviglia et al. 2013). In case of GW_494, for the set of markers we used both STRUCTURE and NEWHYBRIDS uniquely identify this individual as a “pure” wolf, despite the clear phenotypic signs of a hybrid (Fig. 2). More likely, the loss of distinctive phenotypic traits of wolves may be a result of a more distant hybridization, and the resolution of 14 microsatellite markers is insufficient for its identification. For example, Kusak et al. (2018) note that 12 microsatellite STRs were not informative enough for identification of the generations of admixture. The difficulties in determining the classes of hybrids using 11 microsatellite markers were described in detail by Korablev et al. (2021). Obviously, in case of GW_494 the interspecific hybridization took place several generations ago and a wider set of STRs or SNPs is needed to confidently determine that instant. But even analyses at genome-wide level could be insufficient if such event occurred, for example, 20 or more generations ago, as it convincing shown by Galaverni et al. (2017).

In general, the application of STRUCTURE and NEWHYBRIDS in our study produced comparable results, clearly distinguishing wolves from dogs, and mixed individuals. However, the results do not provide grounds for a definite decision regarding the generation of the mixed individuals. Moreover, when critically evaluating the results of studies on the hybridization of wolves and dogs based on

STR, it should be noted that there is a possibility of erroneous identifications of immigrants from surrounding populations as hybrid individuals, as shown by Harmonen and coauthors (2021). In our analysis, there were several individuals, in addition to the phenotypically identified hybrids, that also showed sign of possible mixed ancestry. However, we established a relatively high threshold for q_{wolf} to obtain a more confident dividing of the sample into "purebred" animals and those of mixed origin. Perhaps these individuals could be immigrants, but for a confident decision it is necessary to conduct appropriate tests and it is necessary to expand the analyzed sample set using individuals from other populations. To uniquely identify wolves, dogs, and their hybrids using autosomal STRs, Lorenzini et al. (2022) proposed a multiplex panel of 22 microsatellite markers. Harmoinen et al. (2021) suggest using a 96 SNP panel, arguing that these new markers do not require calibration among laboratories, are capable of detecting wolf-dog hybrids up to third-generation backcrosses to wolves, and demonstrate a high genotyping success rate for all sample types. According to Galaverni et al. (2017) a much more in-depth analysis that traces events that occurred many generations ago is possible using a panel of 170,000 SNPs. In combination with multiple genome-wide ancestry reconstruction methods, it allows the clarification of the patterns and dynamics of admixture even in highly introgressed populations, map the genetic bases of phenotypical indicators of hybridization, identify optimal ancestry-informative markers, and support appropriate management practices (Galaverni et al. 2017). The difficulties we encountered in our study have also convinced us of the need to unify the methods of genetic identification of wolves and hybrids implemented in species monitoring programs, especially when it comes to transboundary populations.

In the studied population, we observed fairly high rates of genetic polymorphism, comparable to those noted earlier in Russian Karelia: $H_o = 0.656$, $H_e = 0.709$, $A = 5.7$ (Aspi et al. 2009), Finland: $H_o = 0.712$, $H_e = 0.697$, $A = 5.9$ (Jansson et al. 2014), the central part of European Russia: $H_o = 0.79$, $H_e = 0.80$, $A = 10$ (Korablev et al. 2021), and on average for some regions of Siberia: $H_o = 0.695$, $H_e = 0.649$ (Talala et al. 2020). It is clear that there can be no direct comparison of these indexes. However, the relatively high level of heterozygosity in wolves from the RK does not mean the population is in good state. It may be due to the specific transit position of the territory, the absence of natural barriers, constant contacts with neighboring populations, diversity of the initial population, but at the same time there may be a possible loss of allelic and mitochondrial diversity (Jędrzejewski et al. 2005; Salado et al. 2022). In Finland, e.g., about 20% of the microsatellite alleles found in historical samples are no longer found in the modern population (Jansson et al. 2014). The heterozygosity indices in

the population we studied demonstrated that the observed heterozygosity $H_o = 0.655$ was significantly ($p < 0.05$) less than the expected $H_e = 0.746$, likely indicating a prevalence of inbreeding. This is corroborated by the coefficient $F_{is} = 0.131$, which is higher compared to the indices previously noted for Karelia in 1995–2000, $F_{is} = 0.094$ (Aspi et al. 2009), and in 1997–2000 $F_{is} = 0.063$ (Åkeson et al. 2022). In Finland, the inbreeding coefficient was also slightly lower in 2007–2009 $F_{is} = 0.108$ (Jansson et al. 2012). Nevertheless, the $F_{is} = 0.131$ that we documented refers to a fairly long period of study (2012–2022), during which the population survived under the steadily increasing human persecution. The F_{is} value in our study is quite similar to the corresponding index for some isolated European wolf populations (Lucchini et al. 2004; Sastre et al. 2011).

Nowadays, more than 60 mitochondrial DNA haplotypes (depending on the length of fragments) have been detected for the Eurasian wolf (Ersmark et al. 2016; Nechaeva et al. 2022). A reduction in diversity from 8 historical to 3 modern mtDNA haplotypes has been found in Finland (Jansson 2013). Currently, only two mtDNA haplotypes have been noted in Karelia, and this level of diversity corresponds to small and isolated populations (Randi et al. 2001; Tirronen et al. 2023). We suspect that the low modern diversity of mtDNA haplotypes in the RK wolf population may be due to the species' history, with periods of intensive hunting in the past and significant population declines. However, neither our study nor previous studies (Aspi et al. 2009) have detected a bottleneck effect.

Some of the key parameters for the wolf population dynamics in Karelia for the periods I (1992–2000), II (2001–2011), III (2012–2022) are the following: average annual numbers 528, 380 and 330 animals; average annual harvest rates 146, 77 and 156 individuals; total numbers of wolves killed 1.312, 847 and 1.718, respectively. The twofold increase in wolf harvest in the third vs the second period affected not only the dynamics (for more information, see Tirronen et al. 2023), but also the genetic structure and diversity of the population. Apparently, a lack of available partners may lead to both close-relative breeding and inter-specific hybridization. Due to intensive hunting, the Karelian population may experience a shortage of breeding individuals, and a sort of "vacuum" is formed over vast areas, which can be filled by dispersal from the west by Finnish, and from the east by Arkhangelsk wolves. Theoretically, this process can make these two different fringes of the same population genetically closer.

One of the key points of this study is the appearance, within a relatively short period of time, of several admixed individuals at once in a population that was previously considered phenotypically "purebred". The assumption of a hybrid origin for three of the four phenotypic hybrids has been confirmed genetically. The results of genetic analysis at

14 loci excluded one individual, but we remain convinced of its admixed lineage. Clarification of the status of this animal and several individuals that are signaling possible introgression is essentially a matter of changing the set of genetic markers and expanding the area of the study.

This study is not just a first attempt to identify hybrids in the Karelian wolf population, but also a valid inquiry into species' response to a high level of direct and intensive human persecution. In our case, it is quite possible that the low diversity of mtDNA haplotypes, an increase in the proportion of inbreeding, and the appearance of admixed animals are consequences of the intensified wolf hunting pressure. As a result, wolves mate both with closely related individuals and in some cases with dogs. Our study can be regarded as empirical evidence supporting the hypothesis that wolves engage interspecific hybridization as a behavioral adaptive mechanism securing the species' survival under extremely unfavorable conditions (Bohling and Waits 2015; Adavoudi and Pilot 2021).

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Data availability No datasets were generated or analysed during the current study.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The research followed all relevant laws and ethical standards. The taking of the animals by hunters was in accordance with the legislation on hunting in the Russian Federation and Republic of Karelia.

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