Wolf-dog hybrids arise: new insight into the eastern fringe of the Northern European wolf population

Konstantin F. Tirronen, Anastasiia S. Kuznetsova

Institute of Biology Karelian Research Centre of the Russian Academy of Sciences, Petrozavodsk, Russia

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***Corresponding author:**
Konstantin Tirronen
konstantin.tirronen@gmail.com

ORCID of the authors:
0000-0002-8208-5460
0000-0002-1034-3088

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ABSTRACT

In study, tissue samples of 35 wolves hunted in Russian Karelia and 12 specimens of dogs were used in 2012-2022 for genetic analysis. Among wolves four individuals (11%) were recognized as wolf-dog hybrids (WDH) according to phenotypic characteristics. The analysis of 11 autosomal STR confirmed the hybrid origin of three, and allowed us to exclude one. At the same time, additionally were identified two more admixed individuals with no phenotypic deviations, arising the total number of hybrids to five (14%). Analysis of CR1 mtDNA in the specified sample set revealed the dog haplotype in one of the presumed phenotypically WDH. The mtDNA haplotype diversity was represented by two haplotypes common in Eurasia. Revealed level of genetic diversity of the population by 11 microsatellite loci was high (He = 0.746; Ho = 0.655; A = 8). However, the observed heterozygosity turned out to be notably lower than expected, and the inbreeding coefficient was also high (Fis = 0.131) and, moreover, increased compared to the previously noted (Aspi et al. 2009). In the past, the wolf population of Karelia was considered as pure wolves, with a moderate level of hunting pressure. The observed genetic processes – interspecific hybridization and increased inbreeding occur against the background of a noticeable arise of the new wave of fight against the wolf in Karelia. This region of some importance in the biogeographic sense – through this territory, the connection of animal populations living in Scandinavia and the Russian Plain, and furthered to Siberia is realized.

INTRODUCTION

In most of the cases phenotype of an animal is an important primary characteristic that allows both a specialist and an ordinary people to determine biological species. Everyone is well aware of the ordinary appearance of a wolf – a gray wolf; at least this characteristic is true for northern Eurasia. Wolfs colors of the Old and New Worlds has a differences, and a black morphs are extremely rare in Eurasia opposite to North America (Bibikov 1985; Yudin 2013; Apollonio et al. 2004; Pilot et al. 2019). It is claimed that the variation of black color in wolves of America appeared as an introgression of dog's genome in the past, and the genetic basis of heredity of this signs is also studied quite well but it seems still under discussions (Anderson et al. 2009; Hedrick 2009). We didn't found mentions in literature of black-colored animals in Fennoscandia. At the February 2021 one black wolf (GW_244) was legally shoot on the south-west of Russian Karelia (RK) near the Russian-Finnish border and in few km of Karelian-Leningrad border (61°19'47" N 29°26'10" E). It is interesting that according to personal communication Ilpo Kojola at southern edge of Finnish-Russian border in Finland there is a transboundary pack in which many, perhaps most individuals are black.

The issue of interspecific hybridization of wolf and dog is acute. Many studies across the world consider 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), hybrids identification (Kusak et al. 2018; Dziech 2021), proposals on the need to unify genetic identification approaches (Randi et al. 2014; Lorenzini et al. 2022) and many others. Within the wolf range in Eurasia a clear differences in knowledge of the population genetic structure are certainly traced. Many European populations have been studied in sufficient detailed 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 (Harmoinen et al. 2021; Hindrikson et al. 2016; Jansson et al. 2014; Pilot et al. 2007, 2018; Smeds et al. 2021; Sundqvis et al. 2001; Vilaça et al. 2023). At the same

time, wolf's range in Russia has been studied weak, and wolf-dog hybridization is discussed in only a few studies (Korablev et al. 2021; Pilot et al. 2019).

In the presented study, we focused on wolves of Russian Karelia. Importance of this population is in ensuring the connectivity of Fennoscandian wolves and population of East European Plain (Åkesson et al. 2021; Harmoinen et al. 2021). Studies of neighboring Finland wolf population and involving samples from the territory of RK indicated the need for further study wolf population genetic of this region (Aspi et al. 2009; Jansson et al. 2012). Having previously discussed the issues of wolf hunting in RK and investigating the diversity of mitochondrial haplotypes, we recorded the appearance of wild wolf-like animal with dog's CR1 mtDNA haplotype (Tirronen et al. 2023). However, there have been no direct researches to study genetic structure and wolf-dog hybridization of RK population. Using this hints and data of wolves with phenotype deviations, we decided to study possible cases of wolf-dog hybridization in RK.

In the present study we tried to evaluate the consequences for wolf population due to increasing of recent hunting pressure in possible manifestation of interspecific hybridization, inbreeding increasing, and changes in genetic diversity and population structure. The tasks were to genetically assess whether there is evidences for admixture between wolves and dogs of Karelia using wild phenotypically deviated canids and randomly assigned sample set.

MATERIAL AND METHODS

Study area and wolf population

All samples were collected at time period of 2012-2022 from the territory of Russian Karelia which situated in the northwestern Russia and covered by taiga forests (Fig. 1). The study area spreading approximately 660 km north to south, and from west to east more than 420 km. Total RK square is 172400 km^2 (without area of White Sea) and more than half of RK is forests stands, almost quarter belongs waterbodies (rivers and lakes) a bit less territory covered by swamped areas, rest belongs agricultural lands, settlements, and industrial areas.

Here we should to give some explanations related to the status of the wolf in Karelia:

1. Population size. Since the beginning of the new millennium, wolf population ranges from about 280 to 450 individuals as of February-March (the period of annual animal counts), i.e. at the end of the hunting season and before the appearance of offspring.
2. Distribution. Wolves inhabit throughout RK, but the population is unevenly distributed – in the north of the region (the northern taiga subzone), the population density is much less than in the south (the middle taiga subzone).
3. Wolf hunting. Since 2000, the annual harvest varies from 45 to 236 specimens and until 2010 it did not exceed 100 sp., since 2012 it has become steadily higher than 100, in 2016 it exceeded 160, and in 2018 it reached an absolute peak of 236 individuals. In total, more than 2,700 wolves have been killed in Karelia since the beginning of the new millennium. A monetary bonus is paid to the hunter for each killed wolf. With the twice increased bounties in 2016, the harvest is grown up; in addition, since 2018, wolf hunters are rewarded in the form of an additional license to hunt moose, which has significantly stimulated wolf hunting.
4. Phenotype. Since bounties for the wolf extermination, each hunted animal undergoes a veterinary examination. During procedures the correspondence of the animal body size characteristics to its approximate age is established, the presence of morphological signs (color

features, head and tail shape, body proportions) corresponding to the biological species – wolf is noted.

Sampling

In this study we used 47 canids samples, 36 of them belong to wild and 11 to domestic animals. Among samples of 36 wild canids, 31 belongs to wolves, four presumably hybrids and one to feral dog. The presented set of wolf samples for genetic analysis n = 35 was randomly selected (with the exception of four phenotypic hybrids), while we tried to select samples that cover the territory of Karelia as uniformly as possible.

Until 2017, samples for genetic analysis accounted for 5-10% of the harvested individuals. Subsequently, in stages, we began to receive up to 85% of annual wolf's harvest in RK. Since for each killed wolf suppose payment of bounties to the hunters, so in controversial case of appearance an individual with atypical "dog" signs, the biomaterial was necessarily sent to our laboratory for additional genetic studies. Thus, samples of four suspected WDH were transferred to us.

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

Muscle tissue or a piece of skin with muscle fibers were taken from killed animals and then were placed in tubes with ethanol and stored at −20°C prior to 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 kennel, where stray or masterless dogs came from settlements and, accordingly, could hypothetically participate in interspecific crossbreeding. Also we used one sample of feral dog (GW_50) trapped in wild on south-west of RK. Dog’s hairs were put in paper bags and stored in dark and cold place prior to analysis.

All samples locations, mtDNA haplotypes, genetic status of animals by analyses of 11 autosomal microsatellites were inserted in Excel. Based on this data a geoinformation system of the experimental territory in a complex with analyzed data was created at QGIS 5.11.2

Microsatellite analysis

We used 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), C09.173, CXX279, C20.253 (Ostrander et al, 1993). The forward primer for each locus was labelled 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 amplified loci 0.5 µl of the molecular size standard GeneScanTM 600 LIZ (Applied Biosystems, USA) was added to PCR products.

Determination of fragment length was performed using capillary electrophoresis on Seqstudio genetic analyzer (Applied Biosystems, USA) following the protocol provided by the manufacturer. Results were analysed in GENEMAPPER v. 4.0 (Applied Biosystems, USA).

Some samples were repeated two times to avoid genotyping errors. The presence of null alleles and stuttering were analysed with MICRO-CHECKER v2.2. (van Oosterhout et al. 2004).
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.
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CR1 mtDNA sequencing

Partial sequences (350 bp) of the hypervariable part of the mitochondrial DNA control-region (mtDNA CR1) (Saccone et al., 1987) were obtained using universal PCR primers Thr-L 15926 5'-CAATTCCCCGGTCTTGTAAACC-3' and DL-H 16340 5'-CCTGAAGTAGGAACCAGATG-3' (Vilà et al, 1999) from wild animals. The amplification of mtDNA control region I fragment was carried out the same way as in microsatellite analysis at annealing temperature 52°C. The determination of nucleotide sequences of the amplified mtDNA region was carried out 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 the 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

The software GENALEX (Peakall and Smouse 2012) was used to calculate the number of alleles per locus, observed heterozygosity (Ho), and expected heterozygosity (He) and test for Hardy–Weinberg equilibrium (HWE) in wolves separately. The inbreeding coefficient (Fis) and their statistical significance were calculated using the software ARLEQUIN v.3.5 (Excoffier and Lischer 2010).
To detect recent bottleneck signature in Karelian wolf population, we used software BOTTLENECK v1.2.02 (Cornuet and Luikart 1996). TPM mutation model was chosen as the most appropriate for microsatellite data and a probability of 95% for single-step mutations with variance of 12 was applied as suggested by S. Piry et al. (1999). As far as we had fewer than 20 loci, so the Wilcoxon’s test was used to check if significant excess of heterozygosity was detected (Luikart and Cornuet 1997). Also mode shift test was used to test if frequency classes based on allele sizes shift from the normal L-shaped distribution indicating of rare allele loss.
To estimate relatedness or relationship between individuals on basis of microsatellite data, we used the computer program ML-RELATE (Kalinowski et al. 2006). Simulation was performed in two types of hypothesis tests.

Analysis of wolf-dog hybridisation

To identify wolf-dog hybrids we used genotypes both from the wild wolf population and dogs. STRUCTURE v2.3.4 (Pritchard et al. 2000) to evaluate the number of genetic clusters (K) in the data and to assign individuals to their likely origin. For identification of hybrid samples, the dataset consisted of all 47 individuals, including wolves (n = 31), dogs (n = 12) and four hybrids (n = 4). 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 done for each K using 1000000 of the Monte Carlo Markov chain (MCMC) and discarding the first 100000 of burn-ins. Then results were 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.
GENETIX v. 4.05 (Belkhir et al. 2004) was used to distinguish wolves, dogs and wolf-dog hybrids on the basis of microsatellite data with factorial correspondence analysis (FCA).
To test if these wolves were related, we calculated the maximum likelihood estimates of relatedness using the program ML-RELATE (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 and dog. The overall data set was processed with 1000000 MCMC sweeps.

RESULTS

Phenotype

According to the phenotype descriptions of the entire set of samples n = 35, four individuals were preliminary assigned to the WDH category. The signs by which these individuals are distinguished from the wolf general set are unequal. GW_119 was a semi-synanthropic animal lived outskirts of a city with a population of about 14 thousand people. On survey, characteristic signs were noted – white claws and white pads. GW_244 a completely black wolf-like animal (Fig. 2) lived in the pack of typical wolves and was shot together with ordinary wolf of the same pack. Sample GW_246 hadn’t an exact description, but was accompanied by a characteristic – "hybrid". Adult female GW_494 hasn’t the black stripes on front legs, no black tip of tail, the overall color tone red-brownish, shape of the head and ears looks like dog breed “Akita inu” (Fig. 2). The sample set in which these four WDH were found belongs to the period 2017-2022 during 1127 wolves were harvested. Thus, the share of the presumed phenotypic hybrids is 0.35%.

CR1 mtDNA

The sequences of the mtDNA control region was obtained for 36 individuals from a wild wolf population (35 wolves and 1 feral dog). Three haplotypes were revealed, the number of polymorphic sites were 8, haplotype diversity Hd = 0,541±0.034 and nucleotide diversity P =

0,00854±0,00060. Among the obtained haplotypes 18 wolves had the haplotype with accession number in GenBank OP503597 (here and further #1) and 16 had the haplotype OP503598 (#2), both were previously noted for RK and common in that region (Tirronen et al. 2023). Two samples had the third haplotype often found in domestic dogs (Canis familaris) and rare in wolves (Ersmark et al. 2016). It was sample GW_246 marked as a "hybrid" and the feral dog GW_50. Information about haplotypes in suspected and identified hybrids is presented in the Table 1.

Genotype errors

All the samples were genotyped using 20 microsatellite markers, but only for 14 loci it was possible to obtain reliable allele length. Six loci were not included to further analysis due to the appearance of false alleles. The program MICRO-CHECKER analyzed 36 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. These loci were excluded from the wolf-dog hybridization analyzes if they also show significant deviation from HWE. Four loci (CPH2, CXX279, CPH5, FH2054) were significantly out of HWE ($P \leq 0.005$) in the pooled data set of 36 samples. As a result, to assess the hybridization of wolves and dogs in the Karelian wolf population, we used a set of 11 loci: AHT137, CPH2, CPH8, CPH4, CPH12, FH2010, FH2096, AHTk211, vWF, FH2001, C09.173 (Ostrander et al. 1993; Shibuya et al. 1994; Holmes et al. 1995; Fredholm and Wintero 1995; Francisco et al. 1996; Lingaas et al. 1997). All loci were used to assess the genetic diversity of the wolf population and its demographic history.

Genetic diversity of wolf population

All microsatellite loci were polymorphic and diversity was relatively high: Ho = 0.655 (SE = 0.022), He = 0.746 (SE = 0.029), A = 8.1 (SE = 0.835) (Table 2). The overall inbreeding coefficient in the studied population was positive and significantly differed from zero (F = 0.131, P = 0.0000). Also the inbreeding coefficient was significantly positive in five loci (CPH4, CXX279, C09.173, CPH5, FH2054). The STRUCTURE analyses according to 11 autosomal STR of 35 samples of wolves and 12 dogs reveal a clear genetic differentiation into two separate groups. An optimal clustering of samples was obtained with K = 2, with a few intermediate individuals of mixed ancestry (Fig. 3). The average q_{wolf} = 0,990, q_{dog}= 0,988.

Population bottleneck

The Wilcoxon’s test for heterozygosity excess was performed for wolf samples except hybrids in two repeat using 11 and 14 microsatellite markers and did not indicate bottlenecks in both cases (P = 0.681 and P = 0.914). Also the allelic frequency distribution for the Karelian population was L-shaped in both cases as expected for populations that have not gone through recent bottleneck. We obtained similar results using 11 or 14 microsatellite loci, which agree with J. Carlsson (2008) and N. Sastre et al. (2011) when they didn't see the differences in using genetic data with null alleles or without.

Genetic identification of WDH

Clustering using Bayesian approaches in Structure software was performed on the entire data set on 11 loci with an increasing number of inferred clusters from K=1 to K=10. ΔK analysis showed the high peak when K=2, the rest K had no peaks (Fig. 3).

Genetic profiles had clearly divided according to their ancestry into the wolf and dog groups with the average q_{wolf} = 0,990 (N=30) and q_{dog}= 0,988 (N=12). Q-value for pure wolves was ranging from 0.960 to 0.996. Five individuals was of mixed ancestry with q_{wolf} ranged between 0.486 (GW_246) and 0.935 (GW_196) and were identified as WDH. They accounted for 14.3% of the wolf's sample. Threshold of $q_{wolf} \geq 0.935$ were established to distinguish hybrids from pure wolfs. Only for one sample GW_246 with 49% probability we can presume as F1 generation (Table 1).

The results of the NEWHYBRIDS analysis (Fig. 3, Table 1) confirmed the results of the STRUCTURE. Individuals (except for GW_244) were assigned according to the classes of origin (wolf, dog or hybrids). GW_244 was classified as a wolf, but with a 16% probability of backcross to wolf. Sample GW_246 was determined as hybrid to be a backcross wolf (72%) or F2 (25%). The rest samples (GW_91, GW_119, GW_196) were assigned to first generation backcrosses to wolf. (Table 1)

Also in the FCA analysis, wolves, dogs and five hybrids clearly distinguished from each other based on the distributions of allele frequencies at 11 microsatellite loci. The sample GW_243 separated somewhat from the cloud of pure wolves (Fig. 4). Previously, in analyzes it was not detected as a hybrid. The black wolf GW_244 identified as hybrid was hunted together with GW_243 at the same pack. It was established that these wolves were not related. A maximum likelihood estimate of relatedness was 0.0072 and relationship was unrelated.

Of the four individuals which had phenotypic deviations from the wolf normal appearance or were marked as a "hybrid", in two cases (GW_119, GW_244) they hybrid status was confirmed only by STR, in one (GW_246) by STR and mtDNA analyses. An individual GW_494 with the dog phenotype was surprisingly recognized as a pure wolf by both used marker classes (Table 1, Fig 3). For this sample (GW_494) we repeated all stages of analysis since DNA extraction in three times. Genetic analysis of microsatellite markers revealed two additional WDH: sample GW_91 and GW_196, without any "wolf" phenotypic deviations.

DISCUSSION

Discussing the results, we will often refer to the comparison with the Finnish wolf population; it is shall to explain why we are doing this:

1. Biogeographically, the "Karelian" and "Finnish" populations represent a single population that does not have geographical barriers and continues further to the east and south, and its division into two populations is rather nominal (Fig. 1) (Boitani 2003; Kojola et al. 2009);
2. 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);
3. At different periods, attempts were made to assess the level of differentiation of these populations using current sets of autosomal STR. Thus, Aspi et al, (2009) using 10, and Jansson et al. (2014) 17 microsatellite markers indicate that the Finnish and Karelian populations are

differentiated, whereas Åkesson et al. (2022) using a set of 26 autosomal STRs did not reveal significant inter-population subdivision.

There are no any notes in literature about the appearance of hybrid individuals among RK wolves on phenotypic grounds (Danilov 2005; Danilov et al. 1979). As well, WDH have not been revealed in previous wolf population-genetic studies based on microsatellites (Aspi et al. 2009; Jansson et al. 2012, 2014). The sample set of RK wolves used for these genetic researches amounted to 37 specimens covering the periods 1995-2000 and 2009-2010. Whole-genome study of Fennoscandian wolf populations also doesn't detect WDH among 15 samples of RK wolves in second decade of 2000s (Smeds et al. 2020). Among 90 RK wolves samples covering period of 2012-2022 used for analysis of CR1 mtDNA an animal with dog ancestry was found at first time (Tirronen et al. 2023). However, the maternal inheritance of mtDNA limiting the possibility of using this method for reliable detection of WDH (Vilà, Wayne 2001).

The WDH could appear due to combination of disruption of the normal population structure of wolves, decreasing of wolf population density with presence of stray or feral dogs, and even deliberate hybridization. Regular and intensive wolf hunting could lead to a break-down of the social structure, rotation of breeding individuals, changes of breeding behaviour, decrease of reproduction success and survival of yearlings, inbreeding, and hybridization (Ryabov 1973; Jędrzejewska et al. 1996; Brainerd et al. 2008; Liberg et al., 2005).

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, among others. In three cases (GW_119, GW_244, GW_246) this assumption was confirmed genetically. Two more individuals (GW_91 and GW_196) were also identified as wolf-dog hybrids during the genetic analysis of a random sample, but they did not have phenotypic features distinguishing them from wolves. This brings us to the important and previously repeatedly stated conclusion that phenotypic traits are not always sufficient to identify a hybrid individual and require confirmation by genetic methods. The dog mtDNA haplotype in GW_246 presume appearance of this WDH as a result of cross female dog and male wolf. The described event of interspecific crossing is interesting and rare. For hybridization of wolves and dogs in wild, more typical sexual disproportion, when alone female-wolf choose a dog as a pair (Hendrickson et al. 2012). The GW_246 sample belongs to the northern taiga subzone of Karelia, where the wolf population density is relatively low. It seems that the combination of a highly sparse population and intensive human persecution were sufficient for appearance of a rare type hybrid (more likely F2) (Table 1, Fig. 3). Another hybrid unusual in its appearance – GW_244 was strikingly different from the average Karelian wolf in completely black coat coloration. The common color of Karelian wolves is characterized by various shades of light and dark gray, often in combination with reddish color that gives a color scheme from almost straw to dark gray, but black wolves have never been found here (Danilov 2005). It is known that black wolves are common in some populations of North America, but the appearance of such individuals in European populations is associated with possible hybridization with dogs (Gipson et al. 2002; Musiani et al., 2007; Caniglia et al. 2013). Interestingly, for this individual, the simulation in STRUCTERE gives $q_{wolf} = 0.923$, which is extremely close to the threshhold value established in our study to distinguish pure wolves and hybrids ($q_{wolf} \geq 0.935$), and NEWHYBRIDS with a 16% probability defines it as a backcross wolf. Obviously, interspecific hybridization in this case took place several generations ago and to confidently determine this moment, the use of a wider set of STR or SNP will be required. In general, the use of functional mutations, in combination to standard non-coding molecular markers, can additionally help

detect hybridization and introgression of genes in wild canine populations (Caniglia et al. 2013). An interesting result was also obtained in the case of GW_494, both STRUCTURE and NEW HYBRIDS for the set of markers we used uniquely identify this individual as a pure wolf, despite the clear phenotypic signs of the hybrid. Theoretically, the loss of distinctive phenotypic signs of wolves may be the result of a more distant dog introgression, and the resolution of 11 microsatellite markers is insufficient for its identification. For example, Kusak et al. (2018) note that the use of 12 microsatellite STRs was not informative enough for identification of the generations of admixture, they only allowed us to determine if an identified hybrids was a backcross with wolves, a backcross with dogs or a wolf with a past dog gene introgression. The difficulties in determining the classes of hybrids using a similar set of microsatellite markers are described in detail by Korablev et al. (2021). In general, the results obtained in our study on STRUCTURE and NEWHYBRIDS are congruous to each other, and clearly distinguish wolves from dogs, and mixed individuals. However, based on the results obtained, we cannot surely judge the status of most all mixed individuals. To uniquely identify wolves, dogs and hybrids between them using autosomal STR, Lorenzini et al. (2022) proposed the multiplex panel of 22 microsatellite markers. Harmoinen et al. (2021) offer to use 96 SNP panel state that these new markers do not require calibration among laboratories, able to detect wolf-dog hybrids up to third-generation backcrosses to wolves and demonstrate a high genotyping success rate for all sample types. The difficulties we encountered in our study also lead us to the idea of the need to unify the methods of genetic identification of wolves and hybrids implemented in species monitoring programs, especially when it comes to transborder populations.

In the studied population, we observe fairly high rates of genetic polymorphism, comparable to those noted earlier in Karelia Ho = 0.656, He = 0.709 и A = 5.7 (Aspi et al. 2009), Finland Ho = 0.712, He = 0.697 и A = 5.9 (Jansson et al. 2014), central part of European Russia Ho = 0.79, He = 0.80 и A = 10 (Korablev et al. 2021) and on average for some regions of Siberia Ho = 0.695, He = 0.649 (Talala et al. 2020). It is clear that there can be no direct comparison of these indexes due to the use of different sample sizes and sets of microsatellite markers in each single study. However, the relatively high level of heterozygosity of wolves of the RK does not mean that the population is welfare. This can be ensured by the specific transit position of the territory, the absence of natural barriers, continuous connection with neighboring populations, the diversity of the initial population, and at the same time against the background of a possible loss of allelic and mitochondrial diversity (Jędrzejewski et al., 2005; Salado et al. 2022). For example, in Finland, according to Jansson et al. (2014), about 20% of microsatellite alleles were not found in the modern population in comparison with historical samples. The indexes of heterozygosity in the studied population demonstrate that the observed heterozygosity Ho = 0.655 is significant ($p < 0.05$) less than the expected He = 0.746, which could presume the predominance of inbreeding. This is proved by the obtained coefficient Fis = 0.131, which increased compared to the indexes previously noted for Karelia in 1995-2000 Fis = 0.094 (Aspi et al. 2009) and in 1997-2000 Fis = 0.063 (Åkesson et al. 2022). In Finland the inbreeding coefficient was also slightly lower in 2007-2009 Fis = 0.108 (Jansson et al. 2012). The obtained Fis is quite similar with the same index for some isolated European wolf populations (Lucchini et al. 2004; Sastre et al. 2011).

Nowadays, more than 60 mitochondrial DNA haplotypes (depends of the length of fragments) have been observed for the Eurasian wolf (Ersmark et al. 2016; Nechaeva et al. 2022). The reduction of diversity from 8 historical to 3 modern mtDNA haplotypes was found out 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 assumed that the modern low diversity of mtDNA haplotypes of RK wolf population may be related to the history of the species – periods of intensification of hunting in the past and significant reduction in numbers. However, in our study, the “bottleneck” effect was not detected, as well as in previous studies (Aspi et al. 2009).

Some crucial population-dynamic parameters of wolves in Karelia for the periods I (1992-2000), II (2001-2011), III (2012-2022): the average annual number 528, 380 and 330 specimens; the average annual harvest rate 146, 77 and 156 individuals; in total 1.312, 847 and 1.718 wolves were extracted, respectively. The twofold increase in wolf harvest in the third period compared to the second affected not only the dynamics of the number (for more information, see Tirronen et al. 2023), but also, the genetic structure and diversity of the population. Apparently, on the background of a lack of partners, both closely related breeding and interspecific hybridization can occur. Due to intensive hunting, the Karelian population may experience a shortage of breeding individuals, and a certain "vacuum" is formed for the rather vast areas, which can be filled by the dispersion from the west by Finnish, and from the east by Arkhangelsk wolves. Theoretically, this can make these two populations genetically closer, although it is difficult to predict how the management of the wolf population of these regions will change in the future, which will largely determine the structures of these populations.

One of the key points of this study is the sudden appearance, in a relatively short period of time, of several WDHs at once in a population that was previously considered conditionally "pure". It is important to note that previously, the composition of relations between wolves and dogs, in the area of the Finnish-Karelian wolf population, exclusively fit into the predator-prey system (Tikkunen, Kojola 2020; Tirronen 2008). An important question arises – does the resulting ratio of pure wolves and hybrids correspond to the real one in the studied population? In our sample set of 35 individuals, four were not random (phenotypical suspected hybrids), so we can ignore them. However, during the genetic analysis of the remaining random sample (n = 31), two individuals turned out to be WDH, which is 6.5%. We refrain from directly transferring the obtained ratio to the entire sample of wolves harvested during this period (n = 1718), considering the statistical power of the sample we examined to be insufficient (n = 31). This ratio is close to that observed in wolf populations in human-dominated landscapes and where man actively interferes in the natural history of wild species (Pacheco et al. 2017; Hindrikson et al. 2012). Our study could be considered as one of empirical confirmation the hypothesis outlined in relation to the canid species about the turning on of interspecific hybridization as a bechavioural adaptive mechanisms allowing the species to survive at appearance of extremely unfavorable conditions (Bohling and Waits 2015; Adavoudi and Pilot 2021).

In undisturbed populations, the core of the population is formed by a family-pack, in which usually only the alpha pair participates in reproduction. In the case of a harvested population, individuals of different ages, social status, and even close kinship can enter into reproduction (Ballard et al. 1987; Mech 1999; Fuller et al. 2003; Yudin 2013). However, even this forced reactions also has its biologically determined limits, a striking example is the observation of wolves on the Isle Royale, when a small and isolated population at a certain point in its existence becomes unable to overcome a complex of intraspecific restrictions and consequences caused by inbreeding depression (Hedrick et al., 2019). An increasing of inbreeding in population has serious demographic consequences, reductions of resistance to parasites and infectious diseases (Liberg et al. 2005; Niskanen et al. 2014). Considering our example, it is quite possible that the low diversity of mtDNA haplotypes, an increase in the proportion of inbreeding and the appearance of interspecific hybrids with a dog are consequences of the intensification of wolf

hurvest. As a result, mating occurs, both with closely related individuals, and in some cases with dogs. Anyway, the presented study is not only the first attempt to identify hybrids in the Karelian wolf population, but also an valid investigation showing the reaction of the species to direct and intense human persecution. In the future, large-scale research, including genomic cross-border studies, will need to answer applied questions about the status of the population and will be extremely important from the fundamental standpoint of studying the processes of microevolution and adaptive capabilities of the species.

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Fig. 1. Wolves in Fennoscandia according to Kaczensky et al., 2021, supplemented by our data for the Murmansk and Karelia regions (A). Samples locations in the Republic of Karelia (B): circles - pure wolves, triangles - wolf-dog hybrids (yellow color is the wolf CR1 mtDNA haplotype, brown - dog CR1 mtDNA haplotype)

Fig. 2. The skin of suspected wolf-dog hybrid GW_494 but genetically recognized as pure wolf and general appearance of wolf-dog hybrid GW_244 (photos provided by local hunting authorities)

Fig. 3. Genetic differentiation of wolves, dogs and WDH: (A) K values (Evanno et al. 2005); bar plotting outputs of the analysis from STRUCTURE (B) and NEWHYBRIDS (C)

Fig. 4. FCA by 11 STR illustrating the genetic distribution of dogs (black dots), wolves (yellow squares), and hybrids (blue dots)

Table 1. Identifications of probabilities for phenotypic and putative hybrids found in the wolf population of Russian Karelia

Table 2. Genetic diversity. Expected (He) and observed (Ho) heterozygosities, number of alleles (A) and inbreeding coefficient (Fis) of 14 microsatellite loci in RK wolf population (n = 35)

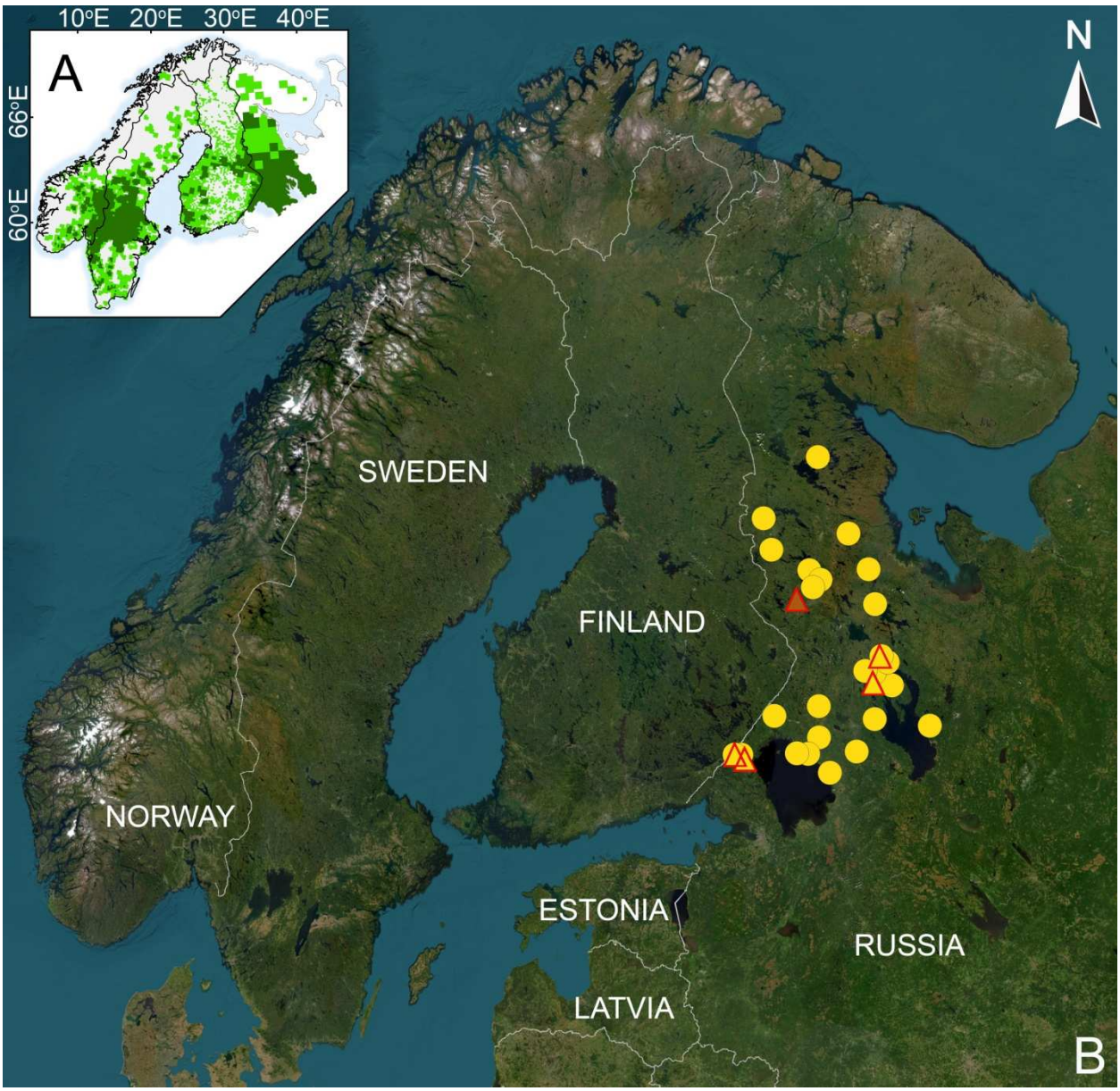

10°E
20°E
30°E
40°E
A
66°E
60°E
N
SWEDEN
FINLAND
NORWAY
ESTONIA
RUSSIA
LATVIA
B

Fig 1

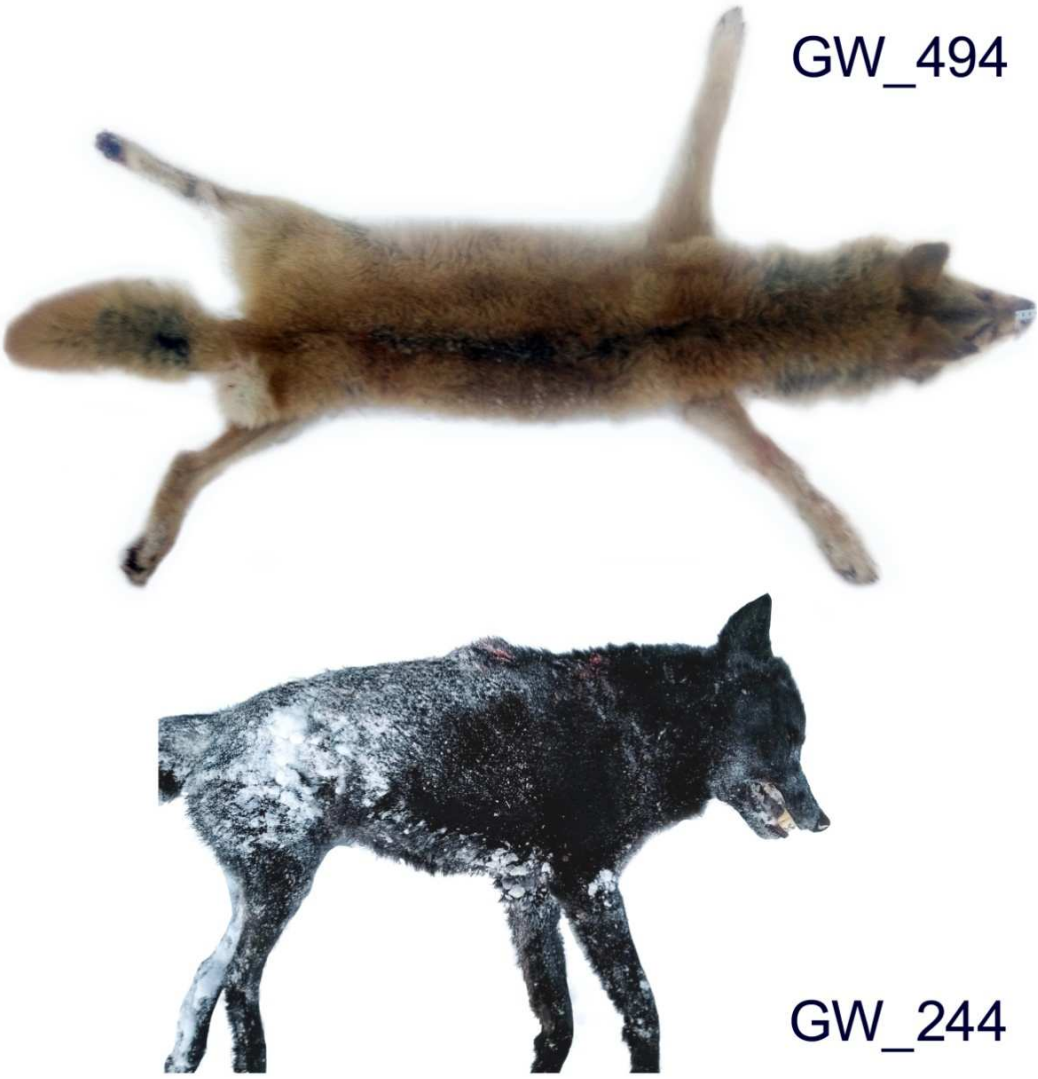

GW_494
GW_244

Fig. 2

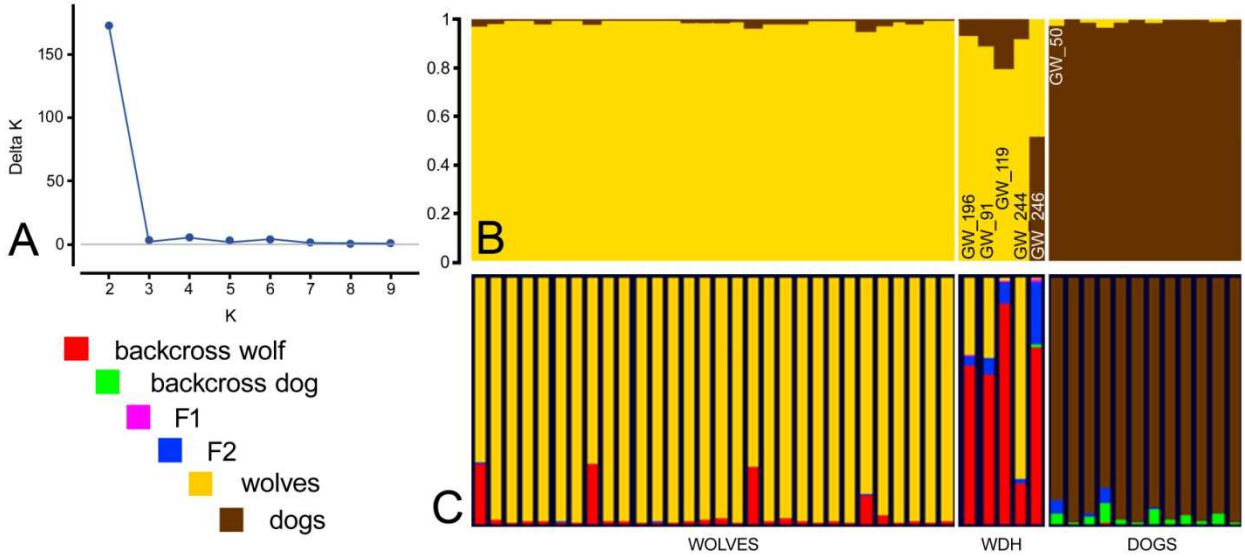

A
Delta K
150
100
50
0
2
3
4
5
6
7
8
9
K
backcross wolf
backcross dog
F1
F2
wolves
dogs
B
1
0.8
0.6
0.4
0.2
0
GW_196
GW_91
GW_119
GW_244
GW_246
GW_50
C
WOLVES
WDH
DOGS

Fig. 3

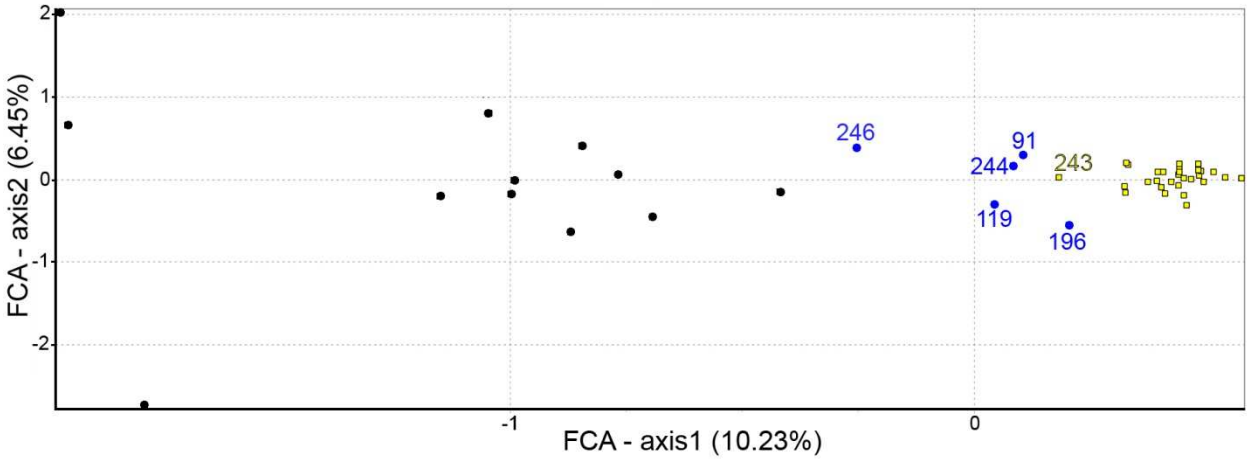


Fig. 4

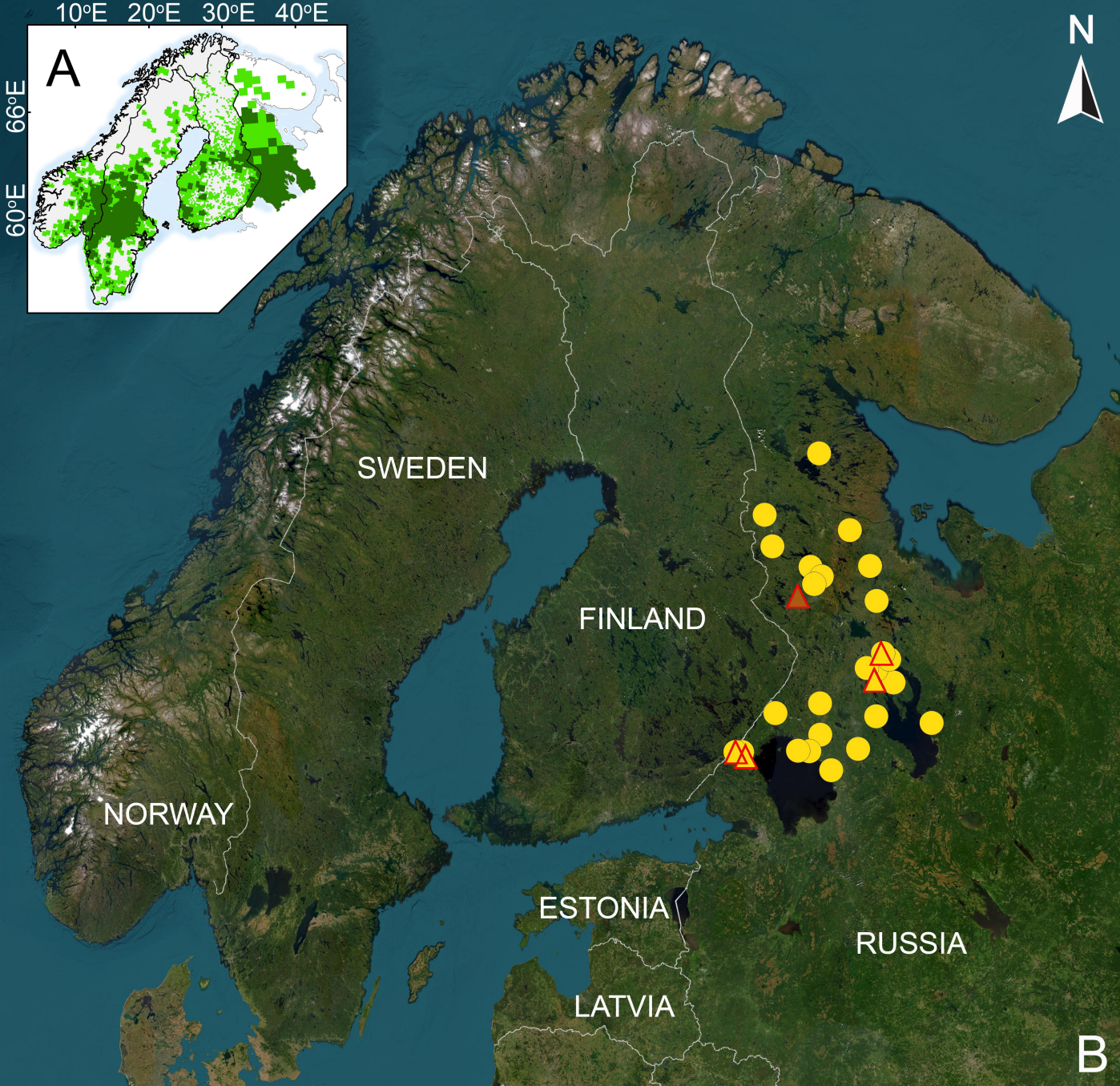
Sample	Year	Location	mtDNA haplotype	Phenotypic trait	STRUCTURE	NEWHYBRIDS					
					q_wolf	Wolf	Dog	F1	F2	BxW	BxD
GW_91	2017	61°19'00" N 29°28'22" E	2	Nothing unusual, “wolf type”	0.9008	0.32683	0.00000	0.00006	0.06681	0.60628	0.00002
GW_119	2018	62°55'44" N 34°28'55" E	1	White claws, white pads	0.7960	0.00986	0.00000	0.00571	0.08911	0.89505	0.00028
GW_196	2020	62°30'54" N 34°14'34" E	2	Nothing unusual, “wolf type”	0.9350	0.31410	0.00000	0.00149	0.03718	0.64708	0.00015
GW_244	2021	61°19'47" N 29°26'10" E	2	Completely black coloration	0.9234	0.81968	0.00000	0.00040	0.01664	0.16312	0.00016
GW_246	2021	63°47'N 31°39'E	dog	Marked as a “WDH” (without details)	0.4856	0.00055	0.00001	0.01290	0.25360	0.71972	0.01323
GW_494	2022	64°16'30"N 34°05'10"E	1	No black tip on tail and black stripes on front legs	0.9940	0.99273	0.00000	0.00000	0.00007	0.00719	0.00000

Table 1

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	

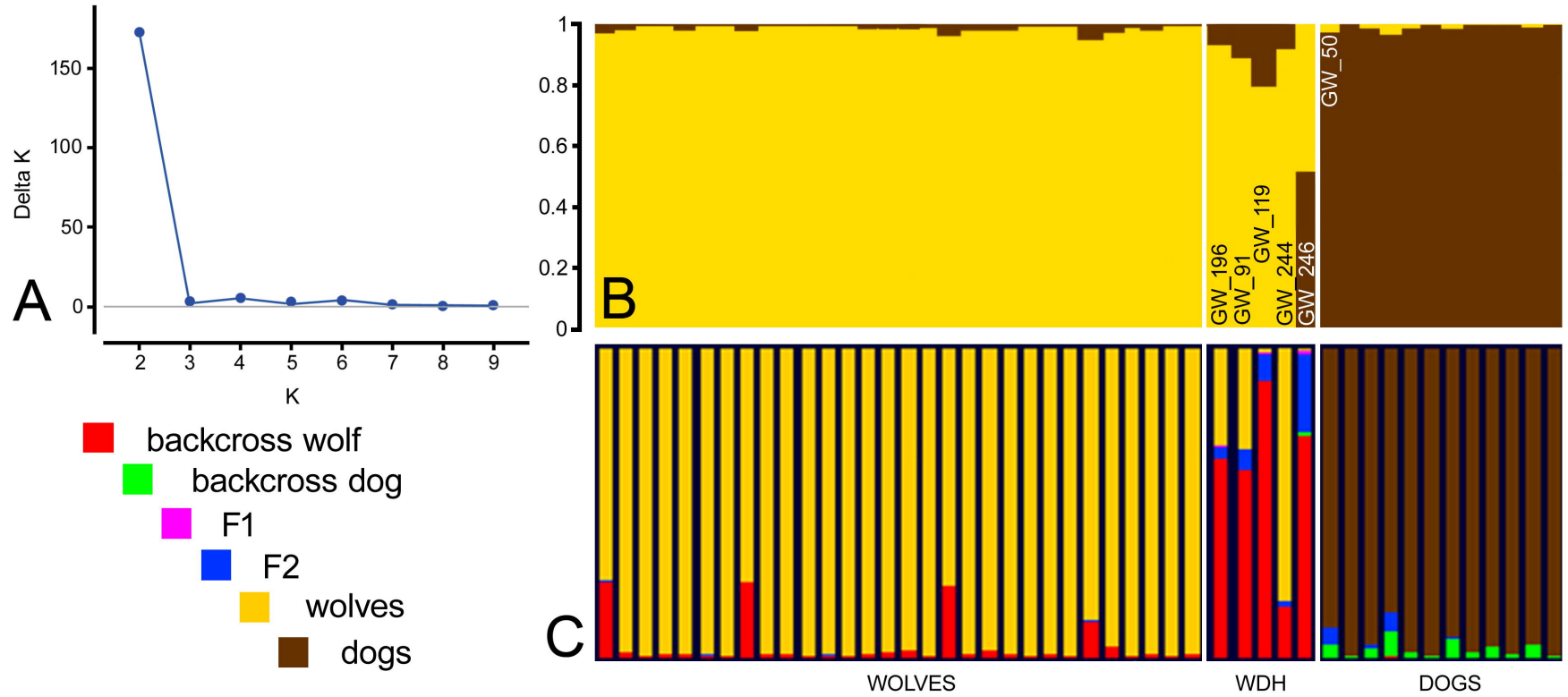
* $p < 0.05$

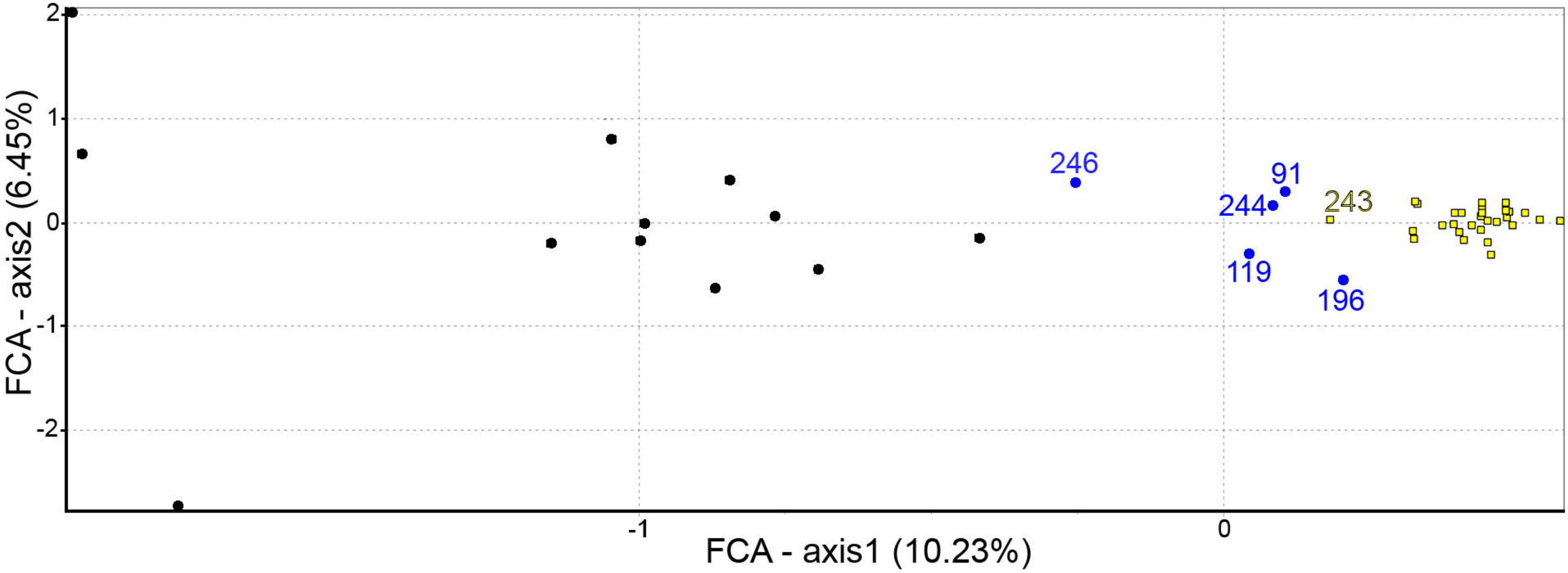
Table 2


10°E
20°E
30°E
40°E
A
66°E
60°E
N
SWEDEN
FINLAND
NORWAY
ESTONIA
RUSSIA
LATVIA
B

GW_494

GW_244


A
Delta K
150
100
50
0
2
3
4
5
6
7
8
9
K
B
1
0.8
0.6
0.4
0.2
0
GW_196
GW_91
GW_119
GW_244
GW_246
GW_50
C
WOLVES
WDH
DOGS
backcross wolf
backcross dog
F1
F2
wolves
dogs


2
1
0
-1
-2
FCA - axis2 (6.45%)
-1
0
FCA - axis1 (10.23%)
246
91
244
243
119
196