Biological Conservation 266 (2022) 109442

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon

Policy analysis

Genome-wide diversity loss in reintroduced Eurasian lynx populations urges immediate conservation management

Sarah Ashley Mueller [a,b,c,*], Stefan Prost [d,e], Ole Anders [f], Christine Breitenmoser-Würsten [g], Oddmund Kleven [h], Peter Klinga [i,j], Marjeta Konec [k], Alexander Kopatz [h], Jarmila Krojerová-Prokešová [l,m], Tomma Lilli Middelhoff [f], Gabriela Obexer-Ruff [g], Tobias Erik Reiners [a], Krzysztof Schmidt [n], Magda Sindičič [o], Tomaž Skrbinšek [p], Branislav Tám [p,q], Alexander P. Saveljev [r], Galsandorj Naranbaatar [s], Carsten Nowak [a,c]

[a] Conservation Genetics Group, Senckenberg Research Institute and Natural History Museum Frankfurt, Clamecystrasse 12, 63571 Gelnhausen, Germany
[b] Institute for Ecology, Evolution and Diversity, Goethe-University Frankfurt, Max-von-Laue-Straße 13, Frankfurt am Main 60438, Germany
[c] LOEWE-Center for Translational Biodiversity Genomics, Senckenberg Research Institutes and Natural History Museums, Senckenberganlage 25, 60325 Frankfurt am Main, Germany
[d] Natural History Museum Vienna, Central Research Laboratories, Burgring 7, 1010 Vienna, Austria
[e] South African National Biodiversity Institute, National Zoological Garden, Pretoria 0184, South Africa
[f] Harz National Park, Oderhaus 1, 37444 Sankt Andreasberg, Germany
[g] KORA, Carnivore Ecology and Wildlife Management, Thunstrasse 31, CH-3074 Muri, Switzerland
[h] Norwegian Institute for Nature Research, Høgskoleringen 9, NO-7034 Trondheim, Norway
[i] Technical University in Zvolen, T.G. Masaryka 24, 960 01 Zvolen, Slovakia
[j] DIANA – Carpathian Wildlife Research, Mládežnícka 47, 974 04 BanskáBystrica, Slovakia
[k] Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia
[l] Institute of Vertebrate Biology of the Czech Academy of Sciences, Květná 8, 603 65 Brno, Czech Republic
[m] Department of Zoology, Fisheries, Hydrobiology and Apiculture, Faculty of AgriSciences, Mendel University in Brno, Zemědělská 1, 613 00 Brno, Czech Republic
[n] Mammal Research Institute, Polish Academy of Sciences, Stoczek 1, 17-230 Białowieża, Poland
[o] Faculty of Veterinary, Medicine University of Zagreb, Heinzelova 55, Zagreb, Croatia
[p] National Zoological Garten Bojnice, Zoological Department, Zámokaokolie 6, 97201 Bojnice, Slovakia
[q] Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Small Animal Science, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia
[r] Department of Animal Ecology, Russian Research Institute of Game Management and Fur Farming, 79 Preobrazhenskaya Str, Kirov 610000, Russia
[s] Mammalian Ecology Laboratory, Institute of Biology, Mongolian Academy of Sciences, 54b Enkhtaivan avenue, Ulaanbaatar 13330, Mongolia

ARTICLE INFO

Keywords:
Conservation genomics
Inbreeding
Large carnivore
Runs of homozygosity
Species translocation
Population management
Reintroduction biology

ABSTRACT

Reintroductions may produce populations that suffer from decreasing genetic diversity due to isolation, genetic drift and inbreeding if not assisted by careful management. To assess the genetic outcomes of reintroductions in large carnivores, we used the Eurasian lynx (*Lynx lynx*) as a case study, which was the subject of several reintroduction attempts over the last 50 years. Although some restocking actions initially appeared successful, lynx recovery has stagnated in recent years. To reveal potential genetic causes of slow lynx recovery in Europe, we examined genome-wide patterns of genetic diversity and inbreeding using single nucleotide polymorphisms (SNPs) in all six successfully reintroduced populations in central Europe, as well as twelve natural populations across Europe and Asia. All reintroduced populations showed lower genetic diversity and elevated levels of inbreeding compared to source and other natural populations. Recent inbreeding is prevalent in all reintroduced populations with varying degrees of severity; the most severe cases are those with the lowest number of founding individuals. Interestingly, we found evidence of lower genetic diversity and recent inbreeding in the source population for five reintroduced populations, begging the question if individuals taken from these source populations can safeguard sufficient genetic diversity for future reintroductions. Given the observed genetic consequences, we advocate for standardized regular genomic assessment of source and target populations as well as

Abbreviations: ROH, runs of homozygosity; RADseq, restriction site associated DNA sequencing; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; LD, linkage disequilibrium.

* Corresponding author at: Clamecystraße 12, 63571 Gelnhausen, Germany.
E-mail address: sarah.mueller@senckenberg.de (S.A. Mueller).

https://doi.org/10.1016/j.biocon.2021.109442
Received 24 April 2021; Received in revised form 2 December 2021; Accepted 23 December 2021
Available online 14 January 2022

individuals prior to release. Our study provides compelling evidence for the serious consequences of founder population size on the genetic diversity of reintroduced large carnivore populations, which has broad implications for their conservation.

1. Introduction

Large carnivores exert a wide range of cascading ecological effects that regulate and maintain ecosystems and can enhance overall biodiversity (Ripple et al., 2014). Many apex predators have become regionally extinct in large parts of their historic ranges, with their return being considered a key step in ecosystem restoration (Lipsey and Child, 2007). One potential way to foster the return of large carnivores is active reintroduction. Numerous reintroduction projects across a wide array of taxa have been conducted to reestablish species to their native ranges (Godefroid et al., 2011; Frosch et al., 2014; Cochran-Biederman et al., 2015; La Haye et al., 2017). Some actions have led to positive results (vonHoldt et al., 2008; Frosch et al., 2014; Moseby et al., 2018; Jiménez et al., 2019), while others failed post-release or required constant restocking (Griffith et al., 1989; Fischer and Lindenmayer, 2000).

Genetic factors can have a major influence on the overall outcome of reintroduction efforts (Frankham, 2009). In the short-term, the genetic composition of released individuals, the size of the founding population and outbreeding depression are major concerns (Keller and Waller, 2002; Hayward and Somers, 2009). Ensuring a sufficient pool of unrelated released individuals is particularly important for species with low reproduction rates (Noss et al., 1996) to avoid inbreeding (Armstrong and Seddon, 2008). Given the high spatial and food requirements as well as potential for human-wildlife conflict, large carnivores are considered more challenging to translocate than other species (Noss et al., 1996; Garrote et al., 2020).

Once a population has been established, isolation and small population size contribute to increasing the risk of inbreeding and reduction of genetic diversity as well as accumulation of deleterious mutations through genetic drift (Whitlock, 2000; Frankham, 2005). Inbreeding is commonly observed in reintroductions with small founding populations (Hayward and Somers, 2009). Inbreeding depression can lower individual fitness resulting in lowered fertility and survival (Keller and Waller, 2002). As a long-term consequence of accumulated inbreeding and reduced genetic variability, reintroduced populations may become more vulnerable to environmental change (Xue et al., 2015) or suffer demographic effects of lower fitness, and face possible extinction (Frankham, 2005).

Modern advances in genetic and genomic methods enable the investigation of these potentially detrimental genetic consequences of reintroductions (Xue et al., 2015). Traditionally, population monitoring is carried out through surveys or DNA-based monitoring using mitochondrial (mtDNA) sequence data and microsatellite analysis (Breitenmoser-Würsten and Obexer-Ruff, 2003; Sindičić et al., 2013; Bull et al., 2016; Krojerová-Prokešová et al., 2019; Mueller et al., 2020). These methods are prone to ascertainment bias and lack of comparability across laboratories, which present serious obstacles to compare genetic diversity across large geographic scales, national borders as well as across multiple populations and generations. Measures of genetic diversity and inbreeding derived from microsatellite markers have been shown to correlate only loosely with genome-wide heterozygosity estimates (Väli et al., 2008). In contrast, genomic methods such as restriction site associated DNA sequencing (RADseq) allow for high resolution genome-wide analysis of genetic diversity and inbreeding (Grossen et al., 2018) and can provide more detailed insights into the potential long-term viability and extent of inbreeding in reintroduced populations. Runs of homozygosity (ROH) analysis, for instance, can be used to uncover recent inbreeding more accurately than traditional heterozygosity estimates that do not take the location of SNPs into account (Kardos et al., 2015; Forutan et al., 2018). Such accurate genowewide inbreeding estimates can be used to inform managers of acute threats to population health and viability resulting from genetic erosion (Kardos et al., 2018; Grossen et al., 2018).

The Eurasian lynx (*Lynx lynx*, Linnaeus 1758) provides a suitable example to study genetic consequences of reintroductions using genome-wide markers due to diverse population histories and demography across the range, including a number of reintroduction attempts. It is a large solitary carnivore; its historical range stretched across the Palearctic from Western Europe to East Asia. During the 19th and 20th centuries, populations in Europe faced extensive persecution and became locally extinct in several regions (Chapron et al., 2014). Today, the Eurasian lynx is considered to have a large, stable population in Russia and central Asia. A stable population is present in Fennoscandia, which underwent a significant bottleneck during the first half -20th century (Hellborg et al., 2002, Pulliainen, 1968, Chapron et al., 2014, Supplementary Table S1). The Baltic population (Poland, Belarus, Lithuania, Estonia and Latvia) is exposed to considerable habitat fragmentation in its western-most part and has decreased in recent years (Supplementary Table S1; Schmidt et al., 2009). The Carpathian population has been the main source of European lynx reintroductions across Europe. The population is considered stable, however, there have been significant population fluctuations in the western part over the last century, including a notable decline in the 1930s, a result of strong hunting pressure, and a quick recovery after legal protection (Hell, 1968; Jamnicky, 1997). Currently, sub-structuring within the West Carpathian Mountains was suggested by genetic as well as coat-pattern analysis (Krojerová-Prokešová et al., 2019; Kubala et al., 2020).

From 1971 to 2006, 17 different reintroduction and translocation projects were implemented to restore populations of this elusive carnivore in Western and Central Europe (Linnell et al., 2009; Idelberger et al., 2021; Molinari et al., 2021). These projects faced a number of challenges and setbacks (see Appendix 1 for more details). Many projects released only a few individuals and could not adequately monitor the population post-release (Linnell et al., 2009). Given early setbacks, only six reintroductions founded populations which experienced demographic growth in years post-release (Fig. 1A). Nearly two decades after the last reintroductions, several populations are undergoing noticeable changes in demography (Appendix 1). Human induced mortality, especially legal and illegal hunting and persecution, has affected several populations negatively (Breitenmoser-Würsten and Obexer-Ruff, 2003; Sindičić et al., 2016; Heurich et al., 2018).

All reintroduced populations are currently monitored, including DNA-based methods. Microsatellite analysis discovered that reintroduced populations display low genetic diversity (Breitenmoser-Würsten and Obexer-Ruff, 2003; Bull et al., 2016; Mueller et al., 2020), some to the point of being in critical status (Sindičić et al., 2013). Given that the reintroduced lynx populations are faced with low genetic diversity and high inbreeding, which affects the species' ability to survive in the long term, we aimed to provide the first genome-wide assessment of reintroduced and natural Eurasian lynx populations. In particular, we aimed to answer the following questions: i) what is the extent of inbreeding and genome-wide genetic diversity loss in reintroduced lynx populations compared to natural populations, and ii) is genetic erosion of the reintroduced populations severe enough to warrant their management through translocation and supplementary measures?

Our data constitute an important baseline for the currently envisioned Eurasian lynx conservation strategy to form a large, connected Central European lynx metapopulation which will be capable of maintaining a high level of genetic variability through gene flow among reintroduced and adjacent natural (i.e. non-reintroduced) populations

(Molinari-Jobin et al., 2010; Bonn Lynx Expert Group, 2021). Based on our results we give recommendations for further conservation management of reintroduced lynx populations in Central Europe. Further, we discuss factors contributing to reintroduction outcomes and, specifically, if exchange of animals among reintroduced Central European populations could enhance levels of genetic diversity.

2. Methods

2.1. Sample collection and DNA extraction

We obtained 308 samples from 14 different countries collected from 2000 to 2019 (Fig. 1, Supplementary Table S2). We sampled all six successfully reintroduced populations within Central Europe (excluding ongoing projects, such as the Palatinate Forest, Germany). Five were sourced from the Slovak Carpathians (Swiss-Alpine (ALP), Swiss-Jura (JURA), North-Eastern Swiss (NE-CH), Bohemian-Bavarian-Austrian (BBA), and Dinaric (DIN)) and one from captive-bred individuals from German and Swedish zoos (HARZ) (Fig. 1A, Appendix 1).

We also sampled the Slovak Carpathians (CARP) to investigate individuals from the source population. We included seven additional individuals that originate from the Polish and Romanian Carpathians, four of which were sequenced by Lucena-Perez et al. (2020) (Fig. 1A, Supplementary Table S2). In addition, we sequenced samples from seven populations identified solely by geographical location, namely North-Eastern Poland (POL), Latvia (LAT), Estonia (EST), Finland (FIN), Norway (NOR), Kirov (KIR), and Mongolia (MON). We included 35 samples from Lucena-Perez et al. (2020) from the Ural, Tuva, Yakutia (YAK), Primorsky Krai (PRIM) and MON populations to comprise a large part of the Eurasian lynx distribution for comparison.

Samples included mainly tissue [251], but also blood [17], dried skin [27], bone [7], hair [2], and feces [2]. For invasive samples, DNA was isolated using the Qiagen Blood and Tissue Kit following the manufacturer's protocols. We added an additional step to treat the samples with RNase A after lysis. The Genomic DNA Mini kit Tissue was used to extract DNA from bone and hair samples and the QIAamp DNA Stool Mini Kit was used to extract DNA from fecal samples, both following the manufacturer's protocols. We chose 190 samples for GBS, which met quality specifications and maintained equal sampling distribution. The extracts were diluted to 10–15 ng/μl to fit sequencing recommendations.

2.2. GBS sequencing

Genomic DNA from the selected 190 samples was converted into nextRAD genotyping-by-sequencing libraries (SNPsaurus, LLC) as in Russello et al. (2015). Genomic DNA was first fragmented with Nextera DNA Flex reagent (Illumina, Inc.), which also ligates short adapter sequences to the ends of the fragments. The Nextera reaction was scaled for fragmenting up to 25 ng of genomic DNA. Fragmented DNA was then PCR amplified with one of the primers matching the adapter and extending 10 nucleotides into the genomic DNA with the selective sequence GTGTAGAGCC. Thus, only fragments starting with a sequence that can be hybridized by the selective sequence of the primer were efficiently amplified. The GBS libraries were sequenced on a HiSeq 4000 with one lane of 150 bp reads (University of Oregon).

Upon receiving the sequencing data, the raw reads were first trimmed to remove adapter sequences as well as stretches of low quality and ambiguous bases using Adapter Removal v2.3.0 (Lindgreen, 2012). At this step, we added bam files of 39 individuals with whole genome sequences from a previous study (Lucena-Perez et al., 2020).

After filtering, the cleaned reads were mapped to the Iberian lynx reference genome (Abascal et al., 2016, https://denovo.cnag.cat/lynx) using BWA-MEM (v 0.7.12-r1039) (Li and Durbin, 2009). We performed SNP calling using samtools (v1.9) (Li et al., 2009) mpileup function. The called raw SNPs were filtered to remove: 1) individual samples with missing data above 65% or with less than 5.0X average coverage; 2) loci with missing data in 30% of the samples, minor allele frequency (MAF) lower than 0.05, or depth lower than 3.0X and higher than 70X; and 3) loci with genotype quality less than 20. In addition to these criteria, the SNPs were also pruned to account for linkage disequilibrium (LD) using $r^2 > 0.8$ in 100 kb windows using bcftools v1.9 (Li et al., 2009).

2.3. Analysis of population structure

We first performed a principal component analysis (PCA) on the entire dataset, and on a subset of natural Carpathian and Carpathian

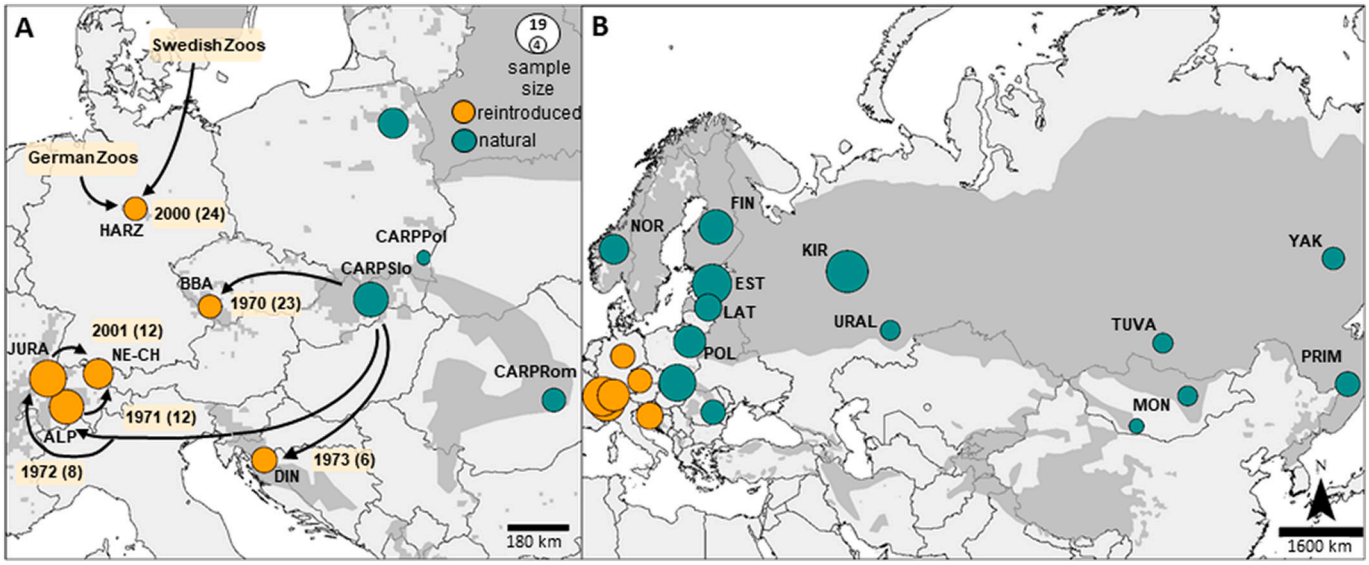

Fig. 1. Sampled populations and reintroduction history of the Eurasian lynx. A) Map of the six sampled reintroduced populations (ALP, JURA, LUNO, BBA, DIN, and HARZ). Sample sizes are shown and ranged from 8 to 18 individuals. The year denotes when reintroduction first began at each respective site (additional translocation years not shown, see Appendix 1) and the minimum number of individuals released in brackets. Arrows show the source population for each respective reintroduction. The Carpathian source population was also sampled. We sampled across the Carpathian Mountains, however, reintroductions were only sourced from CARPSlo. B) Sample locations representing 11 natural populations used in this study. Sample sizes ranged from 6 to 22. Natural populations have been subject to numerous human induced demographic changes over the past 200 years (additional details in Supplementary Table S2).

sourced reintroductions (ALP, JURA, NE-CH, BBA, DIN, CARP) using PLINK (v1.9) (Chang et al., 2015). This method requires no a priori information on populations allowing an unbiased estimation of the general trends in allele frequencies. Next, we calculated genotype likelihoods with the samtools model in ANGSD (v0.930) (Korneliussen et al., 2014) to estimate likelihoods with a SNP p-value of 1e−6. We then used ADMIXTURE (v 1.3.0) (Alexander and Lange, 2011) to infer individual ancestries from the SNP dataset from a K = 1 to K = 18 with 50 repetitions of each K to investigate convergence patterns. We consolidated the runs with CLUMPAK (Kopelman et al., 2015) and estimated the optimal K value based on the Evanno method (Evanno et al., 2005) and the Ln Pr(X|K) method. We also performed hierarchical structure testing, as outlined in Janes et al. (2017) to look for substructuring in identified clusters. We looked at population histories by utilizing Treemix (v1.13) (Pickrell and Pritchard, 2012) to determine a maximum likelihood tree for the sampled populations using the available *Lynx rufus* genome as a root. Treemix can also infer the number of admixture events, so we examined trees that had 0–5 migration events. Lastly, we calculated population F_{ST} values with ANGSD. Here, we used the reference genome as the ancestral, as no ancestral genotypes are available.

2.4. Genetic diversity measures

Tajima's D was calculated with ANGSD, where theta for all loci was generated to calculate an overall Tajima's D statistic. Observed and expected heterozygosity was calculated with the *hierfstat* package in R (Goudet, 2005). Further, we used a Kruskal-Wallis test with post-hoc Bonferroni adjusted p-value to evaluate if there were significant differences in population observed heterozygosity estimates. We utilized STACKS populations (v.2.41) (Catchen et al., 2013) to estimate Pi, and private alleles within each population.

2.5. Inbreeding

We investigated the extent of inbreeding across all populations. We used the filtered SNPs and further filtered for sex-linked markers as these can influence inbreeding estimates (Humble et al., 2020). We first mapped the SNP flanking regions using BWA MEM default parameters to the domestic cat genome as it is assembled to the chromosome level. The 349 loci that were located on the X chromosome were removed from inbreeding analysis (Supplementary Fig. S1). We used ANGSD to create genotype likelihoods and subsequently analyzed individual inbreeding levels. As we suspected inbreeding in reintroduced populations based on available monitoring data, we aimed to use a method that is capable of handling populations that may not fit the assumptions of Hardy-Weinberg equilibrium (HWE) and have low sample sizes (<30 per population). Therefore, we used ngsF (Vieira et al., 2013) to calculate the inbreeding coefficient (*F*) as it is not reliant on allele frequencies, but utilizes an expectation-maximization algorithm that is robust to the uncertainty of assigned genotypes. We calculated 95% confidence intervals from these values using the *dplyr* package in R.

Identifying recent inbreeding through runs of homozygosity (ROH) is particularly important for small, isolated populations and can inform management decisions. ROH analysis can be heavily influenced by SNP density, missing data, MAF and LD filtering, and sequencing depth (Duntsch et al., 2021, Meyermans et al., 2020, Ceballos et al., 2018). Therefore, adequate parameter testing prior to estimating ROH across individuals is required. We used three high quality whole genome re-sequenced samples (LL212_Carp, LL146_Yak, LL112_Prim) from Lucena-Perez et al. (2020) to run parameter testing and re-filtered our SNP dataset. No missing data was allowed and we did not perform MAF or LD filtering as this can hinder accurate ROH estimation (Meyermans et al., 2020). This resulted in 1.2 million SNP sites for ROH estimation. We ran ROH analysis using PLINK testing several different parameter sets based on the sequencing depth and number of loci (Supplementary Fig. S11).

From our dataset including 212 individuals, we excluded individuals with >20% missing data and refiltered the SNP loci without MAF or LD filtering (Duntsch et al., 2021). This resulted in 150 individuals and 21,827 SNP loci for ROH analysis (Supplementary Table S2). We first compared the 3 WGS individuals across different parameters to test the accuracy of ROH analysis in this reduced dataset (Supplementary Fig. S11). We subsequently performed ROH analysis on 150 individuals across a range of parameters. We varied the –homozyg-window-snp and –homozyg-snp thresholds based on recommendations for SNP density (Kardos et al., 2015; Duntsch et al., 2021). We also varied the number of missing loci allowed (–homozyg-window-missing) from 1 to 3. We used the parameters that showed the best concordance to the ROH identified through WGS sequencing to calculate the number and length of ROH across populations. Significant differences in population wide estimates of ROH were evaluated using a Kruskal-Wallis test with a Bonferroni adjusted p-value.

3. Results

3.1. GBS sequencing

Library preparation and sequencing was carried out successfully in 190 samples chosen for sequencing with an average of 656,578 unique reads per sample. Mapping to the Iberian lynx reference genome (PRJEB12609, Abascal et al., 2016) resulted in an average alignment of 95.77%. Three samples mapped below 65%, which were subsequently removed from analysis. Another seven samples exhibited low coverage and seven samples had >65% missing data across SNPs and were removed from analysis. The 39 samples from Lucena-Perez et al. (2020) were already mapped to the reference genome and used in subsequent SNP calling. These remaining 212 individuals formed the basis for our analysis. Sample sizes across populations ranged from 6 to 23 individuals (ALP, 16; BBA, 9; CARP, 22; DIN, 9; EST, 18; FIN, 15; HARZ, 8; JUR, 18; KIR, 19; LAT, 9; NE-CH, 11; MON, 8; NOR, 10; POL, 11; PRIM, 10; TUVA, 6; URAL, 6; YAK, 7). Sample sizes reflect a sufficient number to calculate population genetic statistics based on the number of SNP loci available for analysis (Nazareno et al., 2017). The samples had an average coverage of 18.6X and 14.6% missing data in called loci (Supplementary Fig. S2). After SNP and linkage disequilibrium filtering, 13,525 SNPs were utilized for analysis.

3.2. Population structure

The first axis of the PCA analysis, which explains 34.4% of the variance, separates the Carpathian lineage from the Northern and Siberian lynx lineages (Fig. 2A). The 2nd PCA axis separates the Northern and Siberian lineages. The Harz population appears to be an intermediate between these main clusters. When we add the third axis, explaining 5.2% of the variance, further separation of the Norwegian and Baltic populations is noticeable (Fig. 2B).

Bayesian population structure analysis showed similar results, revealing a high level of population substructuring in both reintroduced and natural populations of Eurasian lynx. Results suggested K = 2 as the optimal value using the Evanno and Ln Pr(X|K) method (Supplementary Table S3). However, given the bias for K = 2 using the Evanno method (Janes et al., 2017) and high number of samples originating from the Carpathian lineage, it is important to evaluate multiple levels of population structuring (Meirmans, 2015, Kalinowski, 2011). When K = 3, the Northern and Siberian lineages separate, in accordance to known trends in Eurasian lynx population structuring (Lucena-Perez et al., 2020). When K = 4, the Swiss reintroductions (ALP, JURA, NE-CH) form a distinct cluster (Fig. 2C), which is separated further in K = 7. Results from PCA analysis on only the Carpathian lineage indicates further evidence for a split between Swiss reintroductions as well as lower levels of genetic drift in the BBA and Dinaric populations (Supplementary

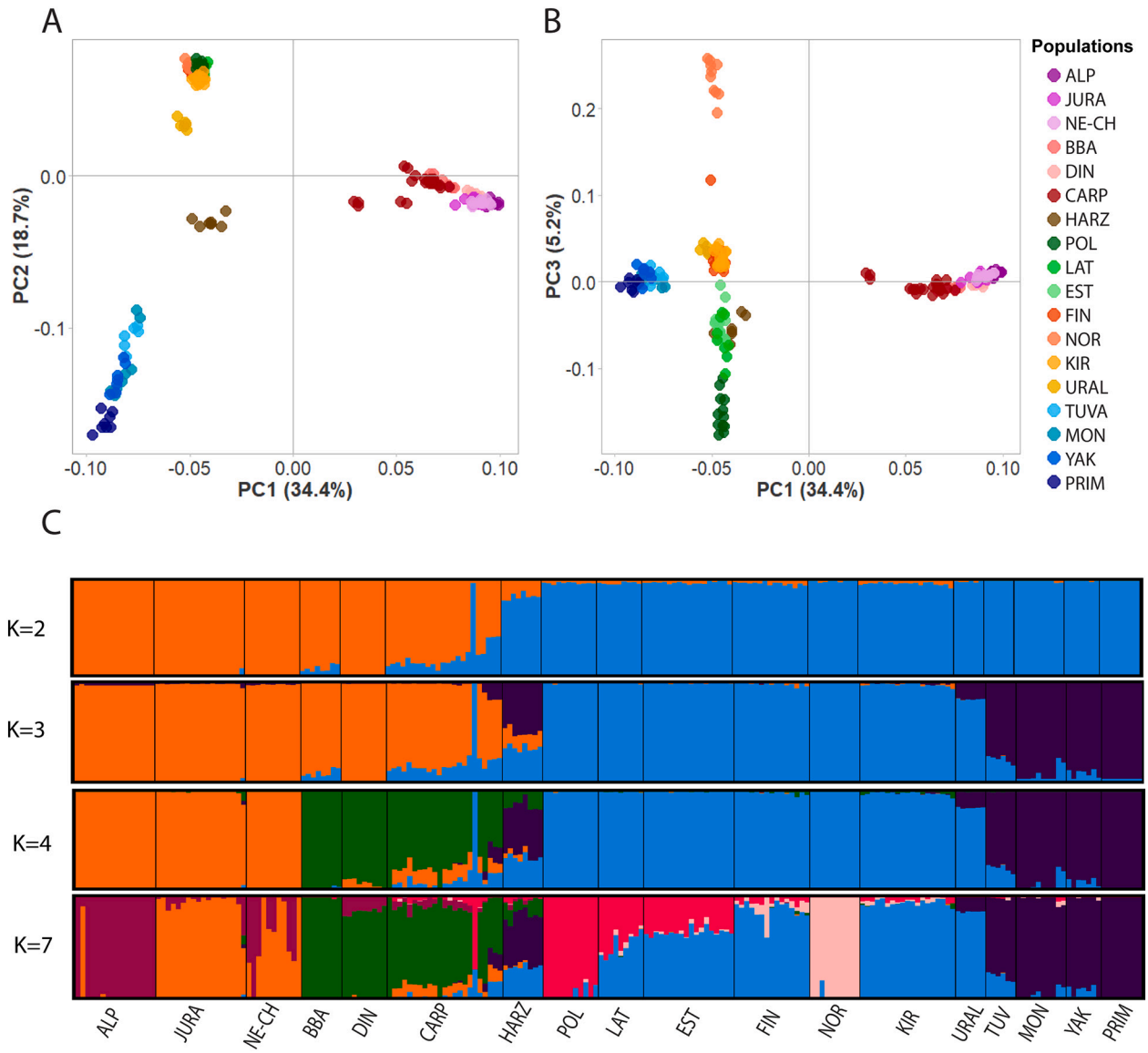

Fig. 2. Relationship between individuals based on 13,525 SNP sites identified from nextRAD sequencing. A) Principal component analysis with the first PC axis separating the Carpathian lineage from the Northern and Siberian lineages. The second axis separates the Siberian from Northern lineage. Notably, the HARZ population represents an intermediate cluster between all three known lineages. The Ural population is also slightly separated from the Northern lineage cluster. Additionally, within the CARP population, there appears to be substructuring based on geographic location (see Supplementary Fig. S3 for more details. B) The third PC axis shows separation among Baltic and Scandinavian populations. The Norwegian samples cluster distinctly from all other Northern lineage samples, and the Baltic region shows increasing genetic drift moving westward. C) Admixture results showing K = 2, K = 3, K = 4, and K = 7, showing population separation. Results generally confirm separations suggested by the PCA and iterative testing (Fig. S4) and multiple Delta K peaks suggest there is a high level of substructuring among natural and reintroduced Eurasian lynx populations.

Fig. S3). K = 7 also identifies substructuring among natural populations, specifically in the Norwegian population. A new cluster is also formed in the Baltic region, partially separating Poland from Latvia and Estonia. Results of iterative testing confirmed this additional substructuring within the two main clusters (Supplementary Fig. S4), further advocating for a high level of substructuring.

Pairwise F_{ST} values revealed similar genetic structuring to PCA and Admixture results (Supplementary Fig. S5). Results from maximum-likelihood phylogenetic analysis supported the separation between known Eurasian lynx lineages: Carpathian, Northern, and Siberian. It placed the HARZ as an intermediate between these lineages, and when treemix considered possible migration events, indications of gene flow between DIN and BBA were suggested (Supplementary Figs. S6 and S7).

3.3. Genomic diversity

Calculations of individual heterozygosity demonstrated significantly lower observed heterozygosity values in reintroduced populations compared to natural populations ($p \leq 0.001$), except for the Harz population (Fig. 3, Supplementary Table S5). Among natural populations, the NOR, and CARP populations had slightly significantly lowered values than TUVA and YAK populations.

3.4. Inbreeding

Inbreeding was first evaluated by calculating F, which revealed inflated inbreeding in reintroduced populations as 95% confidence intervals did not fall below zero (F = 0.40 for reintroduced, F = 0.26 for natural; Supplementary Fig. S8). The lowest signatures of inbreeding

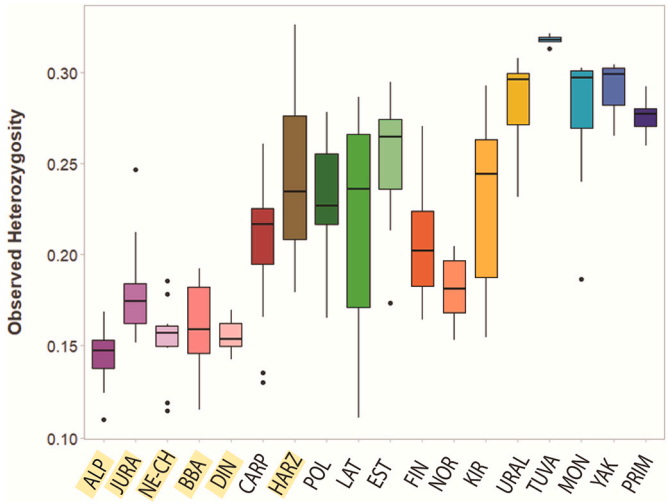

Fig. 3. Observed heterozygosity calculated for 212 Eurasian lynx individuals representing 18 populations; reintroduced populations are highlighted in yellow. The Siberian lineage (blue) shows the highest levels of genetic diversity. Heterozygosity values from populations in the Northern lineage (green, orange and yellow) shows high variability across populations, a result of complex recent demographic histories. The Harz population (brown) shows moderate observed heterozygosity values, with high within population variance. The natural Carpathian population (dark red) shows moderate levels of observed heterozygosity, and reintroductions sourced from the Carpathian Mountains (purple and red) show decreased heterozygosity, with variability among populations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were found in Asian populations.

ROH analysis of LL212, LL146, and LL112 using both the WGS and nextRAD datasets showed similar trends, although chosen parameters can highly influence called ROH and should be considered carefully. We only considered ROH >1 Mb as consideration of smaller segments can lead to erroneous results (Duntsch et al., 2021). The total number of ROH was slightly inflated in LL112 when using the reduced SNP set, however, relative levels between samples remained similar (Supplementary Fig. S11). The number and length of ROH was not significantly impacted by missing data or SNP density (Supplementary Figs. S9, S10).

ROH analysis on 150 Eurasian lynx individuals from the chosen parameters resulted in a total of 5500 ROH segments. Alteration of parameters did not show changes in the overall trends of ROH abundance or length across populations (Supplementary Fig. S12). We identified recent inbreeding in all reintroduced populations, with an increasing trend as the number of released individuals decreases (Fig. 4). Recent inbreeding is significantly higher in reintroduced populations ($p < 0.0001$). The most severe case is the DIN population, although elevated rates were also found in the ALP, JURA, NE-CH, and BBA populations (Fig. 4). When examining the natural Carpathian population significantly more inbreeding is observed in the Western edge of the lynx range ($p = 0.023$, Supplementary Fig. S13).

4. Discussion

We have identified genome-wide patterns of genetic diversity loss across reintroduced lynx populations, not previously possible to assess with microsatellite markers. Further, using two different methods to estimate inbreeding, we showed that reintroduced populations exhibit elevated levels of inbreeding, including recent inbreeding events, which can be detrimental to population viability. These results demonstrate the serious consequences of large carnivore reintroductions, especially in cases with low founding population size. This has wide reaching implications for the conservation prospects of the species.

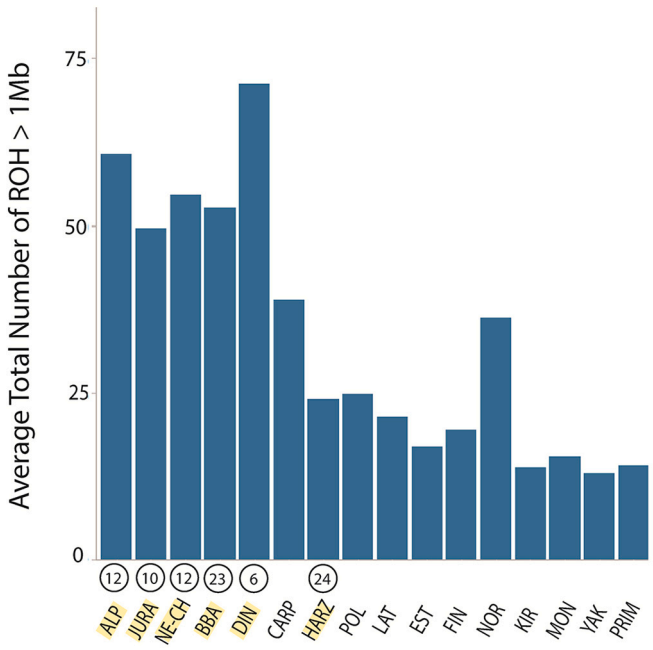

Fig. 4. The average total number of ROH, which are above 1 Mb, in each reintroduced (highlighted in yellow) and natural population calculated from a dataset including 21,827 SNPs. Circled numbers represent the maximum number of released individuals for each reintroduced population. Reintroduced populations show elevated ROH indicating recent inbreeding events, particularly in populations with a low number of founding individuals. Both the CARP and NOR populations also exhibit moderate levels of ROH presence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.1. Genomic consequences in reintroduced populations

Signatures of genetic drift and genetic erosion are prevalent across all reintroduced lynx populations to varying degrees of severity. The significantly lower observed heterozygosity in reintroduced populations indicates that reintroduction bottlenecks, isolation and post-release management have long-term consequences on the genetic composition of populations. Additionally, different degrees of genetic drift across ALP, JURA, NE-CH, BBA and DIN can be explained by differences in the number of founders and demographic histories since reintroduction.

The limited signatures of genetic drift in the BBA population in comparison to the CARP source population can, in part, be attributed to the relatively large total number of released individuals. There were a total of 23–28 individuals released at two sites, with 18 that could have contributed to the founding population (Appendix 1). This was the largest reintroduction of Carpathian lynx from Slovakia. Other reintroduction projects that began around the same time (DIN, ALP, JURA) released between 6 and 12 animals (Čop, 1987; Breitenmoser et al., 1998), and, as expected, genetic drift in these reintroductions was considerably faster.

The captive-sourced HARZ population exhibited higher observed heterozygosity and lower inbreeding than wild sourced reintroductions (Figs. 3 & 4). This can be explained by the mixture of different lineages, which resulted in the inclusion of a higher number of diverse alleles in the population. We must also consider the time since release, as the HARZ population is a comparatively recent reintroduction and there is evidence for ongoing genetic depletion in this population as well (Mueller et al., 2020). Despite these results, we also observed examples of successful preservation of substantial variation within reintroductions of wild sourced individuals. The JURA population, although established with a low number of released individuals (8–10) and showing strong signatures of genetic drift, has maintained a higher level of genetic

variation than other reintroductions from the same source (Fig. 3). We suspect that the JURA population received a more diverse set of genetic founders. Another possibility, which could influence the genetic measures is the sex ratio among genetic founders, which remains unknown for all reintroductions, but could play a role in reintroduction outcomes. The secondary contact with surrounding lynx populations in Swiss Alps and Vosges also cannot be excluded.

Inbreeding plays a key role in determining the health and viability of populations, making its reliable estimation a key goal for conservation planning. Traditional methods of calculating inbreeding are limited due to lack of differentiation between distant and recent events. This distinction is of considerable importance as the latter has a more significant influence on population health and viability as purging the deleterious alleles from the genome has not yet occurred (Kardos et al., 2018; Robinson et al., 2019). Therefore, it is particularly important to quantify ROH burden in reintroduced populations as inbreeding depression is suspected to be more severe in the wild, making early intervention necessary (Ralls et al., 1988).

While estimation of ROH was initially restricted to analysis on whole genomes, recent studies have shown that this measure can be reliably applied to lower density SNP datasets, with careful consideration of parameter settings (Ceballos et al., 2018, van der Valk et al., 2020, Meyermans et al., 2020, Duntsch et al., 2021). Specifically, Duntsch et al. (2021) reported that the number and total length was reliably detected using a RAD sequencing dataset of around 20,000 SNPs, especially in inbred populations. This fits to the results presented here, which show that 21,827 SNP loci accurately matched ROH identified through WGS data in individuals and proved more accurate when individuals had a higher total number of ROH (Fig. S11). Therefore, in line with previous studies, it is key to understand how quality measures can affect results (i.e. missing data and sequencing depth), to perform adequate parameter testing, and to compare when possible to whole genome datasets.

The trend of increasing inbreeding as the number of released individuals decreases (Fig. 4), fits general expectations in reintroduced populations. The Dinaric population has one of the highest rates of inbreeding, in accordance with the low number of founders and the fact that closely related individuals were present among released founders (Koubek and Červený, 1996). Given that the number of genetic founders is likely considerably lower than the number of released individuals in all populations (Mueller et al., 2020), reintroduced populations likely suffer from severe founder effect leading to fast accumulation of inbreeding immediately after the reintroductions, when populations were still extremely small. Given that ROH have been linked to fitness related changes (Xue et al., 2015; Robinson et al., 2019), we can assume that populations with elevated ROH burden are at a higher risk of extinction. While there remains limited data on life history-related traits that can be impacted by inbreeding for reintroduced lynx in Western and Central Europe, we can assume that inbreeding depression may already impact reintroduced populations or will do so in future if they remain isolated.

4.2. Genetic structure of natural Eurasian lynx populations

In general, our results support the known demographic histories of natural Eurasian lynx populations (Lucena-Perez et al., 2020; Rueness et al., 2014) and provide evidence that the genetic consequences of past bottlenecks are still visible despite the recovery of European populations during the last half of the 20th century (Chapron et al., 2014). The lower genetic diversity observed in Finland and Norway can be explained by the bottleneck during the 20th century that affected both populations (Hellborg et al., 2002). The comparatively low ROH values in the Finnish populations despite elevated inbreeding values calculated as a function of heterozygosity (Fig. S8) indicate that while evidence of a past, less severe, bottleneck remains visible, the current inbreeding levels within this population are low. The connection of the Finnish population to the larger Kirov population has likely facilitated gene flow and the partial return to pre-bottleneck composition (Ratkiewicz et al., 2014), similar to trends seen in other large carnivores (Stronen et al., 2013; Kopatz et al., 2014). In contrast, the Norwegian population remains genetically distinct despite evidence of demographic growth (Chapron et al., 2014). Its elevated ROH values and increased inbreeding values suggest that the genetic signatures of severe bottlenecks are visible beyond the point of demographic recovery. In the Baltic region, our results support previous studies suggesting that the northeastern Polish population is partially isolated and has experienced bottlenecks over the last century (Schmidt et al., 2009; Ratkiewicz et al., 2014). This isolation indicates the need to maintain or restore avenues for gene flow within the Baltic region.

4.3. Carpathian population structure

The lynx population from the Carpathian Mountains deserves special attention, as this region served as the founding stock for most reintroductions within the study area and is still the main reintroduction source for ongoing reintroductions (i.e. the Dinaric region and Southwestern Germany). The Carpathian population exhibited a higher total number and longer ROH than other natural populations (except Norway), even when sub-divided by region to account for increased inbreeding at the western-most edge of the distribution (Krojerová-Prokešová et al., 2019). Similar to trends seen in the Norwegian population, this can be partially explained as a genetic signature of the known bottleneck in the 20th century despite subsequent demographic recovery.

The Carpathian lineage has a shared phylogenetic past with the Baltic states until it served as an isolated forest refugium during the last ice age, resulting in the presence of a single haplotype (H4) in this region (Horáček, 1993; Ďurišová et al., 2005; Lucena-Perez et al., 2020). Despite being considered a large, continuous habitat, lynx within the Western Carpathians experienced significant fluctuations in population size over the last century (Hell, 1968; Jamnicky, 1997). Recent studies, along the western edge of the Carpathians revealed elevated levels of inbreeding and suggested population structuring (Krojerová-Prokešová et al., 2019; Kubala et al., 2020). Our results align with this suggesting observed heterozygosity and ROH within the Carpathians appears to be the result of past and recent demographic history. An important area for future investigation is determining the current extent and causes of population structuring across the Carpathian range, specifically in the Polish and Romanian areas.

Given the result of lowered genome-wide diversity and increased inbreeding in Carpathian lynx we stress that individuals captured for future translocation should be given particular care to ensure that unrelated genetically diverse individuals are being chosen for translocation. This would involve genetic testing of individuals at an early stage in the course of reintroduction efforts involving wild-captured lynx.

4.4. Conclusions and implications for conservation

We provide the first comprehensive look at genetic diversity loss and inbreeding across reintroduced and natural populations of Eurasian lynx. Our findings confirm earlier evidence of reduced genetic diversity and elevated inbreeding in reintroduced lynx populations (Breitenmoser-Würsten and Obexer-Ruff, 2003; Sindičić et al., 2013; Bull et al., 2016; Mueller et al., 2020). The results presented here highlight the pressing need for implementing assessments on genetic diversity in the conservation of lynx populations in Europe and especially into future reintroduction efforts. Beyond lynx reintroductions, ensuring a high number of genetically tested founders in large mammal reintroduction programs should be included as an essential part of any species conservation strategy.

Given the current status, reintroduced lynx populations need rigorous management, which incorporates genetic evaluation as a key

component to support population health and viability. A concept for lynx management is currently being developed at both national and European levels (Bonn Lynx Expert Group, 2021). Habitat connectivity between European lynx populations needs to be improved to achieve genetic exchange. Where habitat connectivity and the resulting linkage of populations by natural dispersal cannot be achieved quickly enough, other measures are required. In these cases, we advise further restocking of Eurasian lynx populations, especially those with high levels of inbreeding. We recommend a translocation scheme that meets the principle of "One-migrant-per-generation" (Wang, 2004) until natural gene flow can be established. While wild populations offer an ideal source for translocations, some natural populations already face conservation concerns and monitoring is needed to ensure the wild population would not be harmed by the removal of individuals. When wild sourced individuals are not available, strategies that include translocation between reintroduced populations or reintroduction of captive bred individuals can be evaluated. Importantly, translocation between reintroduced populations should be carefully considered to minimize negative consequences of removing individuals on the existing populations. Different translocation schemes can be tested through simulations to provide implementable long-term strategies.

The ultimate goal of current conservation strategies should focus on creating a viable, genetically diverse metapopulation across the intensively used, cultural landscapes of West and Central Europe, as this is the only way to prevent further genetic erosion in the long term. Recent studies that document natural population expansion (e.g., Mueller et al., 2020) and multiple long distance dispersal events (Gajdárová et al., 2021) within Central Europe show that an interconnected, self-sustaining European lynx meta-population, as envisioned by the Bonn Lynx Expert Group (2021), appears to be an attainable conservation goal even in the fragmented anthropogenic landscapes of Europe.

Data availability statement

Sequencing data associated with this study will be found on Dryad and all codes used in analysis can be found at https://github.com/sa-mueller/Lynx_2021.

CRediT authorship contribution statement

S.A.M., T.E.R., S.P., C.B.-W. and C.N. designed the study. All co-authors contributed samples, S.A.M., J.K.-P., P.K. and M.S. carried out DNA extraction and preparation for HTS. S.A.M. analyzed the data. S.A.M. and C.N. wrote the manuscript and all authors were involved in revision and editing the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Funding for this study was generated through the wildlife genetics analysis service at the Senckenberg Conservation Genetics Section, Gelnhausen, Germany. Additional funds came from the Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz of the German Federal State of Hesse (Center for Translational Biodiversity Genomics; LOEWE-TBG). S.A.M is funded through the DAAD. Collection of samples in Russia and Mongolia was funded by the National Science Centre, grant number: 2014/15/B/NZ8/00212 and FP7 People: Marie-Curie Actions, grant number: PIRSESGA-2009-247652. We thank everyone involved in collecting samples as this constitutes a tremendous amount of work and our study would not be possible without it.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocon.2021.109442.

References

Abascal, F., Corvelo, A., Cruz, F., et al., 2016. Extreme genomic erosion after recurrent demographic bottlenecks in the highly endangered iberian lynx. Genome Biol. https://doi.org/10.1186/s13059-016-1090-1.

Alexander, D.H., Lange, K., 2011. Enhancements to the ADMIXTURE Algorithm for Individual Ancestry Estimation. https://doi.org/10.1186/1471-2105-12-246.

Armstrong, D.P., Seddon, P.J., 2008. Directions in reintroduction biology. Trends Ecol. Evol. https://doi.org/10.1016/j.tree.2007.10.003.

Bonn Lynx Expert Group, 2021. Recommendations for the conservation of the eurasian lynx in Western and Central Europe. Conclusions from the workshop of the "Bonn lynx expert group" in Bonn, Germany, 16–19 June 2019. Cat News Special Issue 14, 78–86.

Breitenmoser, U., Breitenmoser-Würsten, C., Capt, S., 1998. Re-introduction and present status of the lynx in Switzerland. Hystrix Ital. J. Mammal. https://doi.org/10.4404/hystrix-10.1-4118.

Breitenmoser-Würsten, C., Obexer-Ruff, G., 2003. Population and conservation genetics of two re-introduced lynx (Lynx lynx) populations in Switzerland – a molecular evaluation 30 years after translocation. Environ. Encount. 58, 51–55.

Bull, J.K., Heurich, M., Saveljev, A.P., Schmidt, K., Fickel, J., Förster, D.W., 2016. The effect of reintroductions on the genetic variability in eurasian lynx populations: the cases of bohemian-bavarian and Vosges-palatinian populations. Conserv. Genet. https://doi.org/10.1007/s10592-016-0839-0.

Catchen, J., Hohenlohe, P.A., Bassham, S., Amores, A., Cresko, W.A., 2013. Stacks: an analysis tool set for population genomics. Mol. Ecol. https://doi.org/10.1111/mec.12354.

Ceballos, F.C., Joshi, P.K., Clark, D.W., Ramsay, M., Wilson, J.F., 2018. Runs of homozygosity: windows into population history and trait architecture. Nat Rev Genet 19 (4), 220–234. https://doi.org/10.1038/nrg.2017.109.

Chang, C.C., Chow, C.C., Tellier, L.C., Vattikuti, S., Purcell, S.M., Lee, J.J., 2015. Second-generation PLINK: rising to the challenge of larger and richer datasets. GigaScience. https://doi.org/10.1186/s13742-015-0047-8.

Chapron, G., Kaczensky, P., Linnell, J.D.C., 2014. Recovery of large carnivores in Europe's modern human-dominated landscapes. Science (New York, N.Y.). https://doi.org/10.1126/science.1257553.

Cochran-Biederman, J.L., Wyman, K.E., French, W.E., Loppnow, G.L., 2015. Identifying correlates of success and failure of native freshwater fish reintroductions. Conserv. Biol. https://doi.org/10.1111/cobi.12374.

Čop, J., 1987. Propagation Pattern of Re-introduced Population of Lynx (Lynx lynx L) in Yugoslavia (1973 Slovenia—Kocevsko) and its Impact on the Ungulate Community.

Duntsch, Laura, Whibley, Annabel, Brekke, Patricia, Ewen, John G., Santure, Anna W., 2021. Genomic data of different resolutions reveal consistent inbreeding estimates but contrasting homozygosity landscapes for the threatened Aotearoa New Zealand hihi. Mol. Ecol. https://doi.org/10.1111/mec.16068.

Ďurišová, A., Kaminská, Ľ., Kozlowski, J.K., 2005. Pleistocene large mammals. In: Svoboda, J.A. (Ed.), Pleistocene Environments and Archeology of the Dzeravá skala cave, Lesser Carpathians, Slovakia. Krakow, pp. 169–204, 1 - 226.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. https://doi.org/10.1111/j.1365-294X.2005.02553.x.

Fischer, J., Lindenmayer, D., 2000. An assessment of the published results of animal relocations. Biol. Conserv. https://doi.org/10.1016/S0006-3207(00)00048-3.

Forutan, M., Ansari Mahyari, S., Baes, C., Melzer, N., Schenkel, F.S., Sargolzaei, M., 2018. Inbreeding and runs of homozygosity before and after genomic selection in north american Holstein cattle. BMC Genomics. https://doi.org/10.1186/s12864-018-4453-z.

Frankham, R., 2005. Genetics and extinction. Biol. Conserv. https://doi.org/10.1016/j.biocon.2005.05.002.

Frankham, R., 2009. Genetic considerations in reintroduction programmes for top-order, terrestrial predators. In: Hayward, M., Somers, M.J. (Eds.), Reintroduction of Top-order Predators. Wiley-Blackwell, Chichester, UK, Hoboken, NJ, pp. 371–387.

Frosch, C., Kraus, R.H.S., Angst, C., et al., 2014. The genetic legacy of multiple beaver reintroductions in Central Europe. PLoS One. https://doi.org/10.1371/journal.pone.0097619.

Gajdárová, B., Belotti, E., Bufka, L., Duľa, M., Kleven, O., Kutal, M., et al., 2021. Long-distance Eurasian lynx dispersal – a prospect for connecting native and reintroduced populations in Central Europe. Conserv Genet. https://doi.org/10.1007/s10592-021-01363-0.

Garrote, Germán, Fernández-López, Javier, Rojas, Eva, López, Guillermo, Simón, Miguel Angel, 2020. Planning the peninsula-wide recovery of the Iberian lynx: identification of favourable habitat areas. Mammalia 84 (5), 413–420. https://doi.org/10.1515/mammalia-2019-0052.

Godefroid, S., Piazza, C., Rossi, G., et al., 2011. How successful are plant species reintroductions? Biol. Conserv. https://doi.org/10.1016/j.biocon.2010.10.003.

Goudet, J., 2005. Hierfstat, a package for r to compute and test hierarchical F-statistics. Mol. Ecol. Notes 5 (1), 184–186. https://doi.org/10.1111/j.1471-8286.2004.00828.x.

Griffith, B., Scott, J.M., Carpenter, J.W., Reed, C., 1989. Translocation as a species conservation tool: status and strategy. Science (New York, N.Y.). https://doi.org/10.1126/science.245.4917.477.

Grossen, C., Biebach, I., Angelone-Alasaad, S., Keller, L.F., Croll, D., 2018. Population genomics analyses of european ibex species show lower diversity and higher inbreeding in reintroduced populations. Evol. Appl. https://doi.org/10.1111/eva.12490.

Hayward, M., Somers, M.J., 2009. Reintroduction of Top-order Predators. Wiley-Blackwell, Chichester, UK, Hoboken, NJ.

Hell, P., 1968. Population density of the Lynx in the Czechoslovakian carpathians. In: Kratochvíl, J. (Ed.), Recent distribution of the Lynx in Europe. Přírodovědné práce ústavů Československé akadémie věd v Brně 2, 5–6, pp. 57–64, 1–74.

Hellborg, L., Walker, C.W., Rueness, E.K., et al., 2002. Differentiation and levels of genetic variation in northern european lynx (Lynx lynx) populations revealed by microsatellites and mitochondrial DNA analysis. Conserv. Genet. https://doi.org/10.1023/A:1015217723287.

Heurich, M., Schultze-Naumburg, J., Piacenza, N., et al., 2018. Illegal hunting as a major driver of the source-sink dynamics of a reintroduced lynx population in Central Europe. Biol. Conserv. https://doi.org/10.1016/j.biocon.2018.05.011.

Horáček, I., 1993. Obratlovčí fauna Slaninové jeskyně (Turnianske Podhradie, časť Háj) (Vertebrate fauna in Slaninové cave). In: Lamiová-Schmiedlová, M., Mačala, P. (Eds.), Východoslovenský pravek 4. Archeologické ústav SAV Nitra, Košice, pp. 31–35.

Humble, E., Paijmans, A.J., Forcada, J., Hoffman, J.I., 2020. An 85K SNP array uncovers inbreeding and cryptic relatedness in an antarctic fur seal breeding colony. G3: Genes, Genomes, Genetics. https://doi.org/10.1534/g3.120.401268.

Idelberger, S., Back, M., Ohm, J., Prüssing, A., Sandrini, J., Huckschlag, D., Krebühl, K., 2021. Reintroduction of Eurasian lynx (Lynx lynx carpathicus) in the Palatinate Forest, Germany. Cat News Special Issue 14, 38–42.

Jamnicky, J., 1997. Hunting of lynx (Lynx lynx L.) and wild cat (Felis silvestris Schreb.) in Slovakia one hundred years ago. In: Folia Venatoria - Polovnicky Zbornik - Myslivecky Sbornik (Slovak Republic).

Janes, Jasmine K., Miller, Joshua M., Dupuis, Julian R., Malenfant, René M., Gorrell, Jamieson C., Cullingham, Catherine I., Andrew, Rose L., 2017. The K = 2 conundrum. Mol. Ecol. 26 (14), 3594–3602. https://doi.org/10.1111/mec.14187.

Jiménez, José, Nuñez-Arjona, Juan Carlos, Mougeot, Francois, Ferreras, Pablo, González, Luis Mariano, García-Domínguez, Francisco, 2019. Restoring apex predators can reduce mesopredator abundances. Biol. Conserv. 238, 108234. https://doi.org/10.1016/j.biocon.2019.108234.

Kalinowski, S.T., 2011. The computer program STRUCTURE does not reliably identify the main genetic clusters within species: simulations and implications for human population structure. Heredity 106 (4), 625–632. https://doi.org/10.1038/hdy.2010.95.

Kardos, M., Åkesson, M., Fountain, T., et al., 2018. Genomic consequences of intensive inbreeding in an isolated wolf population. Nat. Ecol. Evol. https://doi.org/10.1038/s41559-017-0375-4.

Kardos, M., Luikart, G., Allendorf, F.W., 2015. Measuring individual inbreeding in the age of genomics: marker-based measures are better than pedigrees. Heredity. https://doi.org/10.1038/hdy.2015.17.

Keller, L., Waller, D.M., 2002. Inbreeding effects in wild populations. Trends Ecol. Evol. https://doi.org/10.1016/s0169-5347(02)02489-8.

Kopatz, A., Eiken, H.G., Aspi, J., Kojola, I., Tobiassen, C., Tirronen, K.F., Danilov, P.I., Hagen, S.B., 2014. Admixture and gene flow from Russia in the recovering northern european brown bear (Ursus arctos). PLOS ONE 9 (5), e97558.

Kopelman, N.M., Mayzel, J., Jakobsson, M., Rosenberg, N.A., Mayrose, I., 2015. Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. Mol. Ecol. Resour. https://doi.org/10.1111/1755-0998.12387.

Korneliussen, T.S., Albrechtsen, A., Nielsen, R., 2014. ANGSD: analysis of next generation sequencing data. BMC Bioinformatics. https://doi.org/10.1186/s12859-014-0356-4.

Koubek, P., Červený, J., 1996. Lynx in the Czech and Slovak Republics. Institute of Landscape Ecology of the Academy of Sciences of the Czech Republic.

Krojerová-Prokešová, J., Turbaková, B., Jelenčič, M., et al., 2019. Genetic constraints of population expansion of the carpathian lynx at the western edge of its native distribution range in Central Europe. Heredity. https://doi.org/10.1038/s41437-018-0167-x.

Kubala, J., Gregorová, E., Smolko, P., Klinga, P., Iľko, T., Kaňuch, P., 2020. The coat pattern in the carpathian population of eurasian lynx has changed: a sign of demographic bottleneck and limited connectivity. Eur. J. Wildl. Res. https://doi.org/10.1007/s10344-019-1338-7.

La Haye, M.J.J., Reiners, T.E., Raedts, R., Verbist, V., Koelewijn, H.P., 2017. Genetic monitoring to evaluate reintroduction attempts of a highly endangered rodent. Conserv. Genet. https://doi.org/10.1007/s10592-017-0940-z.

Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics (Oxford, England). https://doi.org/10.1093/bioinformatics/btp324.

Li, H., Handsaker, B., Wysoker, A., 2009. The sequence alignment/map format and SAMtools. Bioinformatics (Oxford, England). https://doi.org/10.1093/bioinformatics/btp352.

Lindgreen, S., 2012. AdapterRemoval: easy cleaning of next-generation sequencing reads. BMC Res. Notes. https://doi.org/10.1186/1756-0500-5-337.

Linnell, John, D.C., BreitenmoserUrsBreitenmoser-WrstenChristineOdden, J., 2009. Recovery of Eurasian Lynx in Europe: What Part has Reintroduction Played? and practice series, no. 5), S. Wiley-Blackwell (Conservation science, Chichester, UK, Hoboken, NJ, pp. 72–91.

Lipsey, M.K., Child, M.F., 2007. Combining the fields of reintroduction biology and restoration ecology. Conserv. Biol. https://doi.org/10.1111/j.1523-1739.2007.00806.x.

Lucena-Perez, M., Marmesat, E., Kleinman-Ruiz, D., et al., 2020. Genomic patterns in the widespread eurasian lynx shaped by late quaternary climatic fluctuations and anthropogenic impacts. Mol. Ecol. https://doi.org/10.1111/mec.15366.

Meirmans, P.G., 2015. Seven common mistakes in population genetics and how to avoid them. Mol. Ecol. 24 (13), 3223–3231. https://doi.org/10.1111/mec.13243.

Meyermans, R., Gorssen, W., Buys, N., Janssens, S., 2020. How to study runs of homozygosity using PLINK? A guide for analyzing medium density SNP data in livestock and pet species. BMC Genomics 21 (1), 94. https://doi.org/10.1186/s12864-020-6463-x. S.

Molinari, P., Breitenmoser, U., Černe, R., Fuxjäger, C., Weingarth, K., Ryser, A., Molinari-Jobin, A., 2021. The contribution of stepping-stone releases for enhancing lynx distribution. Cat News Special Issue 14, 46–49.

Molinari-Jobin, A., Marboutin, E., Wölfl, S., et al., 2010. Recovery of the alpine lynx Lynx lynx metapopulation. Oryx. https://doi.org/10.1017/S0030605309991013.

Moseby, K.E., Lollback, G.W., Lynch, C.E., 2018. Too much of a good thing; successful reintroduction leads to overpopulation in a threatened mammal. Biol. Conserv. https://doi.org/10.1016/j.biocon.2018.01.006.

Mueller, S.A., Reiners, T.E., Middelhoff, T.L., Anders, O., Kasperkiewicz, A., Nowak, C., 2020. The rise of a large carnivore population in Central Europe: genetic evaluation of lynx reintroduction in the Harz Mountains. Conserv. Genet. https://doi.org/10.1007/s10592-020-01270-w.

Nazareno, Alison G., Bemmels, Jordan B., Dick, Christopher W., Lohmann, Lúcia G., 2017. Minimum sample sizes for population genomics: an empirical study from an Amazonian plant species. Mol. Ecol. Resour. 17 (6), 1136–1147. https://doi.org/10.1111/1755-0998.12654. S.

Noss, R.F., Quigley, H.B., Hornocker, M.G., Merrill, T., Paquet, P.C., 1996. Conservation biology and carnivore conservation in the Rocky Mountains. Conserv. Biol. https://doi.org/10.1046/j.1523-1739.1996.10040949.x.

Pickrell, J.K., Pritchard, J.K., 2012. Inference of population splits and mixtures from genome-wide allele frequency data. PLoS Genet. https://doi.org/10.1371/journal.pgen.1002967.

Pulliainen, E., 1968. The lynx population in Finland. Acta. Sc. Nat.Brno. 2, 27–34.

Ralls, K., Ballou, J.D., Templeton, A., 1988. Estimates of lethal equivalents and the cost of inbreeding in mammals. Conserv. Biol. https://doi.org/10.1111/j.1523-1739.1988.tb00169.x.

Ratkiewicz, M., Matosiuk, M., Saveljev, A.P., et al., 2014. Long-range gene flow and the effects of climatic and ecological factors on genetic structuring in a large, solitary carnivore: the eurasian lynx. PLoS One. https://doi.org/10.1371/journal.pone.0115160.

Ripple, W.J., Estes, J.A., Beschta, R.L., 2014. Status and ecological effects of the world's largest carnivores. Science (New York, N.Y.). https://doi.org/10.1126/science.1241484.

Robinson, J.A., Räikkönen, J., Vucetich, L.M., et al., 2019. Genomic signatures of extensive inbreeding in isle royale wolves, a population on the threshold of extinction. Sci. Adv. https://doi.org/10.1126/sciadv.aau0757.

Rueness, E.K., Naidenko, S., Trosvik, P., Stenseth, N.C., 2014. Large-scale genetic structuring of a widely distributed carnivore—the Eurasian lynx (Lynx lynx). PLoS One. https://doi.org/10.1371/journal.pone.0093675.

Russello, M.A., Waterhouse, M.D., Etter, P.D., Johnson, E.A., 2015. From promise to practice: pairing non-invasive sampling with genomics in conservation. PeerJ. https://doi.org/10.7717/peerj.1106.

Schmidt, K., Kowalczyk, R., Ozolins, J., Männil, P., Fickel, J., 2009. Genetic structure of the eurasian lynx population in North-Eastern Poland and the Baltic states. Conserv. Genet. https://doi.org/10.1007/s10592-008-9795-7.

Sindičić, M., Gomerčić, T., Kusak, J., Slijepčević, V., Huber, Đ., Frković, A., 2016. Mortality in the Eurasian lynx population in Croatia over the course of 40 years. Mamm. Biol. https://doi.org/10.1016/j.mambio.2016.02.002.

Sindičić, M., Gomerčić, T., Polanc, P., et al., 2013. Kinship analysis of dinaric lynx (Lynx lynx) population. Šumarski List 137 (1–2), 43–49.

Stronen, A.V., Jedrzejewska, B., Pertoldi, C., Demontis, D., Randi, E., Niedzialkowska, M., Pilot, M., Sidorovich, V.E., Dykyy, I., Kusak, J., Tsingarska, E., Kojola, I., Karamanlidis, A.A., Ornicans, A., Lobkov, V.A., Dumenko, V., Czarnomska, S.D., 2013. North-south differentiation and a region of high diversity in european wolves (Canis lupus). PLoS One 8 (10), e76454.

Väli, U., Einarsson, A., Waits, L., Ellegren, H., 2008. To what extent do microsatellite markers reflect genome-wide genetic diversity in natural populations? Mol. Ecol. https://doi.org/10.1111/j.1365-294X.2008.03876.x.

van der Valk, T., Gonda, C.M., Silegowa, H., et al., 2020. The genome of the endangered dryas monkey provides new insights into the evolutionary history of the vervets. Mol. Biol. Evol. https://doi.org/10.1093/molbev/msz213.

Vieira, F.G., Fumagalli, M., Albrechtsen, A., Nielsen, R., 2013. Estimating inbreeding coefficients from NGS data: impact on genotype calling and allele frequency estimation. Genome Res. https://doi.org/10.1101/gr.157388.113.

vonHoldt, B.M., Stahler, D.R., Smith, D.W., Earl, D.A., Pollinger, J.P., Wayne, R.K., 2008. The genealogy and genetic viability of reintroduced yellowstone grey wolves. Mol. Ecol. https://doi.org/10.1111/j.1365-294X.2007.03468.x.

Wang, J., 2004. Application of the one-migrant-per-generation rule to conservation and management. Conserv. Biol. 18 (2), 332–343.
Whitlock, M.C., 2000. Fixation of new alleles and the extinction of small populations: drift load, beneficial alleles, and sexual selection. Evolution. https://doi.org/10.1111/j.0014-3820.2000.tb01232.x.
Xue, Y., Prado-Martinez, J., Sudmant, P.H., 2015. Mountain gorilla genomes reveal the impact of long-term population decline and inbreeding. Science (New York, N.Y.). https://doi.org/10.1126/science.aaa3952.