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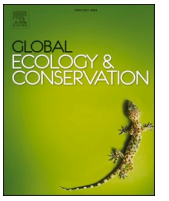

Long-term genetic monitoring of a reintroduced Eurasian lynx population does not indicate an ongoing loss of genetic diversity

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ABSTRACT

Where reintroduced wildlife populations are considered as vulnerable this is generally due to limited founder size and isolation. While many of these populations show low levels of genetic diversity, little is known about the temporal patterns of genetic diversity loss and the role of initial founder effects vs. ongoing genetic drift. Here we analysed genotype data from 582 Eurasian lynx samples from the reintroduced Bohemian-Bavarian-Austrian population (BBA) over a time span of 35 years, representing approximately 13 generations. Two-wave reintroduction of lynx from at least two distinct West-Carpathian areas resulted in relatively high start-up of genetic diversity. After the initial decline when the population lost about a quarter of its genetic diversity compared to the Carpathian source population, the genetic diversity and effective population size remained almost unchanged over the next 20 years. Despite confirmed isolation of BBA and thus absence of gene flow, we detected relatively low inbreeding during the two recent decades within the slightly increasing population size, which may have prevented ongoing loss of genetic diversity. Given the current status of BBA, we do not support genetic reinforcement to maintain its long-term viability; but urge the importance of facilitating gene flow with neighbouring lynx populations through an improvement of landscape connectivity and by strengthening law enforcement as well as the prevention of illegal killings. A sound genetic monitoring alongside regular camera trap-based monitoring of population size, health status and reproduction is pivotal to decide on future conservation interventions.

1. Introduction

Species reintroductions, i.e., human-mediated attempts to re-establish extirpated species within parts of their former range, have become a common practice in conservation efforts (Armstrong and Seddon, 2008; Ottewell et al., 2014; Scandura et al., 2014; Boitani and Linnell, 2015). As the primary purpose of any reintroduction is to establish a self-sustaining population (IUCN/SSC, 2013), not only the establishment but also the long-term persistence of reintroduced population should be assessed to evaluate the success of such effort (Armstrong and Seddon, 2008). In many cases reintroductions fail right from the beginning because the original threatening processes (illegal killing, predation, competition, poor habitat quality) have not been controlled and/or improved sufficiently (Linnell et al., 2009; Stier et al., 2016). Next important driver of post-release population growth that account for success or failure of reintroductions is an intrinsic nature of organisms, including their genetic architecture (Hall et al., 1997; Tracy et al., 2011; Fautley et al., 2012). Regarding the long-term viability of a reintroduced population, the reduction of genetic diversity over time can pose a threat, as genetic diversity is essential for adaptive response to changing environmental conditions (Frankham, 2005; Tracy et al., 2011; Chaturvedi et al., 2021).

Stochastic evolutionary processes, such as genetic drift, are more pronounced in small and isolated populations that have undergone a bottleneck or that have been founded by only a few individuals (Wright, 1931; Crow and Kimura, 1970). Such populations are just a small subsample of an original population, where alleles, mainly those with low frequency, are lost by mere chance (so-called *founder effect*). Moreover, small population size is associated with an increased probability of breeding between close relatives, therefore increasing rates of inbreeding (Frankham, 2005; Neaves et al., 2015). Without gene flow from neighbouring populations, the mean level of inbreeding may increase over time leading to a further loss of fitness (*inbreeding depression*), reducing the long-term viability of a population (Frankham et al., 2002; Dobrynin et al., 2015). In the worst scenario, genetic diversity loss and inbreeding may result in population extinction in a process known as *extinction vortex* (Gilpin and Soulé, 1986).

Despite the obstacles resulting from low population size, some small populations have been able to survive for long periods with low genetic variation. One of possible mechanisms explaining their persistence involves purging of deleterious mutations, which can result in a reduction of genetic load (Wang et al., 1999). Purifying selection is facilitated by inbreeding as it increases homozygosity of partially recessive deleterious mutations, and enables their removal from the population, thus preventing inbreeding depression (Xue et al., 2015; Hedrick and García-Dorado, 2016; Robinson et al., 2018). The empirical evidence of purging of deleterious mutations in small populations is scarce, as it can be revealed with the help of genomic data, the use of which has only been emphasized in recent years (Grossen et al., 2020; Khan et al., 2021; Kleinman-Ruiz et al., 2022; Westbury et al., 2018; Xue et al., 2015). Other mechanisms compensating for the lack of genetic variation in small populations involve genome rearrangement after bottleneck (Carson, 1990), or phenotypic plasticity produced by epigenetic variation (Thorson et al., 2017). Among carnivores, intriguing examples demonstrating the long-term persistence of a population with severely reduced levels of genetic diversity, but no strong signs of inbreeding depression are the Channel Island fox (*Urocyon littoralis*; Robinson et al., 2016, 2018), or the brown hyena (*Parahyaena brunnea*; Westbury et al., 2018). Some examples among other mammals include the pygmy hog (*Porcula salvania*; Liu et al., 2020), the Alpine ibex (*Capra ibex*; Grossen et al., 2020) or the eastern gorilla (*Gorilla beringei*; Xue et al., 2015).

As the above-listed examples illustrate, predicting the impact of inbreeding and impoverished genetic diversity on a population persistence is not always straightforward. More accurate viability predictions in small populations would require the consideration of demographic history, temporal changes in neutral and functional genetic diversity as well as a genome-wide assessment of mutational load (Liu et al., 2020; Kleinman-Ruiz et al., 2022; García-Dorado and Caballero, 2021).

Historically inbred populations appear to be more tolerant of genetic diversity loss (Robinson et al., 2018; Kleinman-Ruiz et al.,

2022). Examples for this are large carnivores, which often persist in small effective population sizes due to low population density as a result of large territory sizes (Khan et al., 2021; van der Valk et al., 2021). Populations of the Eurasian lynx (*Lynx lynx*), for instance, appeared to be once quite homogeneous, but started to diverge during the Pleistocene, around 100 Kya (Lucena-Perez et al., 2020). This east-west differentiation (Rueness et al., 2014) peaked during the Last Glacial Maximum (LGM) and was accompanied by a continuous decline of population sizes, especially in the European part of the species range. Climatic oscillations during the Late Pleistocene and Holocene and human persecution from the 15th century led to fragmentation, isolation, or extirpation of lynx populations in most of Europe (Lucena-Perez et al., 2020). Low population sizes and geographical isolation likely gave rise to the current genetic differentiation among European populations (Hellborg et al., 2002; Schmidt et al., 2009; Ratkiewicz et al., 2012; Lucena-Perez et al., 2020).

A particularly extreme situation is that of lynx populations in Central and Western Europe (the Alpine, Jura, Vosges-Palatinate, Dinaric, and Bohemian-Bavarian-Austrian population), which were established by reintroductions in the 1970s and 1980s after a long period of species' absence due eradication in the 18th and 19th century. These reintroduced populations were founded by a small number of lynx from the West Carpathians, in some cases involving closely related individuals (Koubek and Červený, 1997). All these newly established populations are largely isolated and small-sized (less than 200 or even fewer than 100 individuals), consequently classified as "endangered" or "critically endangered" in the IUCN Red List of Threatened Species (von Arx, 2020).

The Bohemian-Bavarian-Austrian lynx population (hereafter: BBA) was founded between 1970 and 1973 when five to seven lynx of mostly unknown origin (two males and one female were from the Slovak Carpathians) were released in the Bavarian Forest in Germany, close to the border of the Czech Republic. A few years later, between 1982 and 1989, this first release was followed by a reintroduction of 17 lynx from Slovakia into the Bohemian Forest in the Czech Republic (Červený and Bufka, 1996; Volfová and Toman, 2018). However, it is not known if any of the descendants of the first reintroduction survived at the time of the second reintroduction. Throughout its existence, the population has been strictly protected within its distribution range and together with an absence of genetic monitoring programs a collection of historical samples was very challenging due to a limited number of lynx specimens in museum and private collections. Recent camera-trapping data indicate that the size of BBA has been slightly increasing, at least in its core area of the two neighbouring national parks (Šumava and Bavarian Forest in the Czech Republic and Germany, respectively; Palmero et al., 2021). A population size was estimated in 2018/19 (Wölfl et al., 2020) of 123 (99−146) independent animals (adults and subadults).

The first assessment of genetic diversity in BBA was done by Bull et al. (2016) with 12 microsatellite loci, based on only 12 individuals from a small part of BBA distribution range (NP Bavarian Forest). The study found reduced genetic diversity compared to native populations. Surprisingly, they also identified one individual as admixed with the Northern lynx (*L. l. lynx*), thus questioning the presumed demographic isolation of BBA. Recently, Mueller et al. (2022) confirmed the reduced genome-wide diversity within the reintroduced European lynx populations, including BBA. While the finding of somewhat reduced genetic diversity by above mentioned studies is not surprising due to reduced founder numbers and no or low connectivity, there is a lack of long-term data on the temporal dynamics of genetic diversity loss in isolated reintroduced populations; for instance, if populations lose genetic diversity gradually or if allelic diversity stabilizes following the initial founder event, along with a growing population. Understanding the temporal dynamics of genetic diversity change could inform conservation strategies aiming to halt or reduce genetic diversity loss in reintroduced populations.

To evaluate the impact of the founder effect and presumed long-term isolation on the genetic architecture of BBA, we collected samples from historical specimens as well as from the current population. By covering more than three decades of existence of BBA we aimed (i) to assess the rate of supposed decrease in heterozygosity and allelic richness as well as the accumulation of inbreeding over time, and (ii) to analyse the extent of the founder effect through comparison of the current level of neutral genetic diversity with the source Carpathian population and some other native and reintroduced lynx populations in Europe. Besides this, we calculated the current effective population size to quantify the magnitude of genetic drift and inbreeding (iii) and checked for possible immigration events that may have impacted levels of genetic diversity over time (iv). Based on our findings we discuss the implications for the conservation of this enigmatic large carnivore.

2. Materials and methods

2.1. Sampling

A total of 582 samples from BBA (Table 1) were collected between "lynx years" 1984 and 2019 (lynx year = period from 1 May till

Table 1
Genotyping success and error rates for different types of samples. N = number of genotyped samples together with DNA extracts, N successfully genotyped samples (%), Individuals = number of unique individuals (* three individuals were sampled by both, invasive and non-invasive samples), mean percentage per locus allelic dropout (ADO), mean percentage per locus false allele (FA), mean quality index (QI).

SAMPLE TYPE	N	N successfully genotyped (%)	Individuals	ADO (%)	FA (%)	QI
non-invasive	524	173 (33)	71 *	19.3	1.4	0.86
historical	15	11 (73)	11	15.4	0.9	0.79
invasive	43	42 (98)	42 *	0.0	0.3	0.98
together	582	226 (39)	121	11.9	0.9	0.88

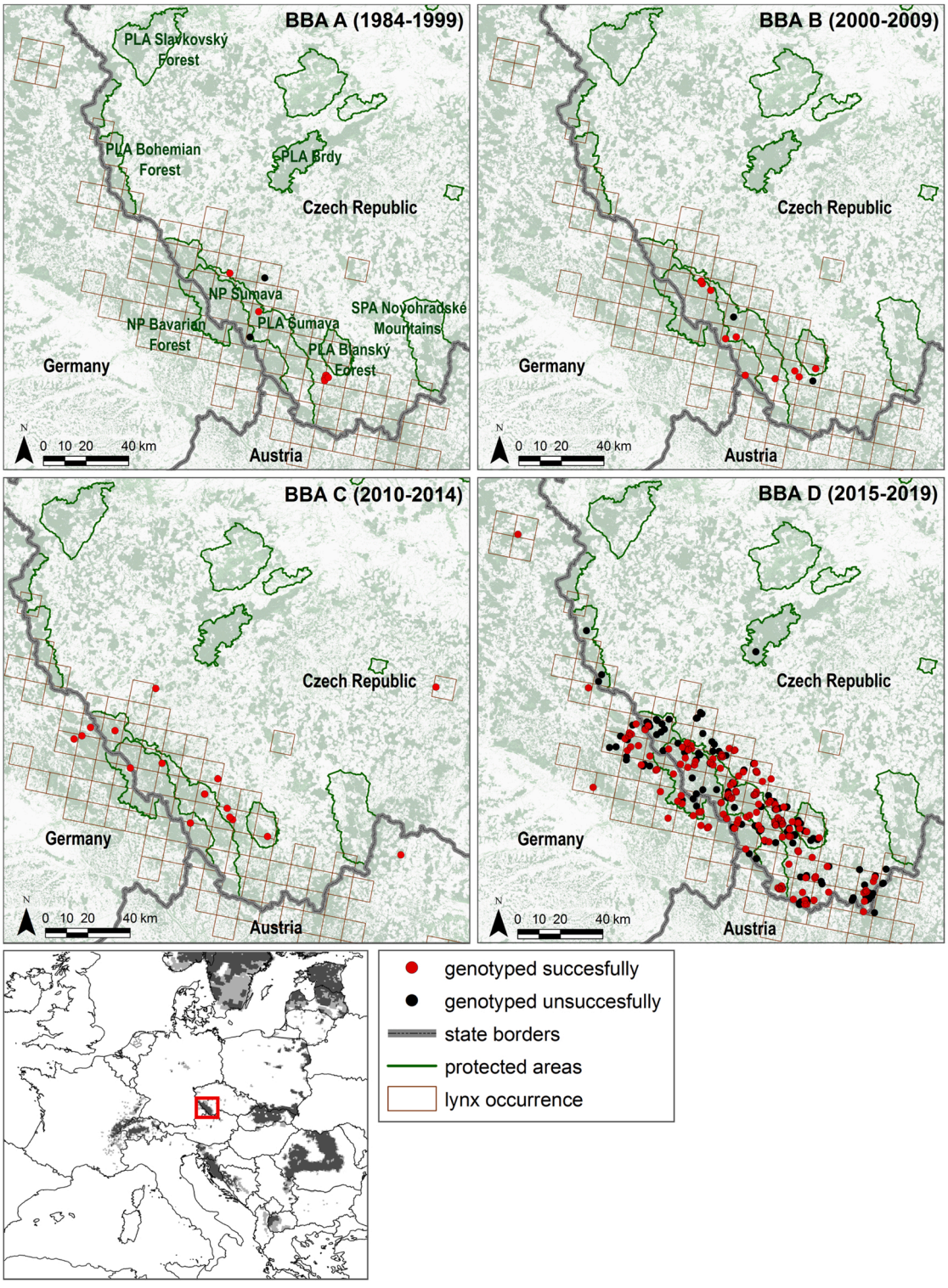

Fig. 1. Study area with sample locations divided into four time periods: BBA A, B, C, and D. In the case of samples from A and B period the coordinates were estimated based on the location name. The distribution range of BBA (brown squares) is displayed according to IUCN Red List Mapping 2012-2016 (Kaczensky et al., 2021). As background, we used the forest type cover layer from © Copernicus Land Monitoring Service (2018), European Environment Agency (EEA), European Union.

30 April of the following year with regard to lynx breeding cycle; Zimmermann et al., 2005). Samples analysed included 524 recent non-invasive samples: hairs (348), scat (138), urine marking from snow or grass (31), saliva traces from lynx prey (4), blood in a lynx track (2), and a castor bean tick (*Ixodes ricinus*) engorged with lynx blood (1). In addition, analysed samples included 43 recent invasive samples: muscle (41), blood (1), and tissue from a found skull (1). Further, 15 historical samples (tissue material from skull (10), from skeleton without skull (1), dried skin (4)) from specimens deposited in museums and private collections were also collected. For 13 out of 582 samples the DNA was extracted in two other labs: 10 DNA extracts used by Hollerbach et al. (2018) were provided by the Centre for Wildlife Genetics, Senckenberg Research Institute and Natural History Museum Frankfurt in Germany, and 3 DNA extracts were provided by the University of Veterinary Medicine, Vienna.

Muscle samples were taken from animals killed by traffic, poached, or from kittens found dead, and one blood sample was taken from an individual found alive after a vehicle collision. Non-invasive samples were collected during snow-tracking or alongside camera-trapping survey, especially at the marking sites.

2.2. DNA extraction and microsatellite genotyping

Microsatellite genotyping was performed in two steps. Firstly, we followed the same protocols of sample storage, DNA extraction, amplification conditions, and fragment analysis of 15 microsatellite loci for individual identification and the amelogenin marker for sex determination as described in Krojerová-Prokešová et al. (2019). Samples that failed to amplify at three or more loci during amplification of the first two independent four-loci multiplexes as well as samples with more than two missing loci out of the first 15 loci were discarded. Consensus genotypes were assigned to the same individual with a maximum of two mismatching loci. In the second step, successfully genotyped samples were analysed at an additional set of five microsatellite loci (see Table S1 for more details). In both steps, we followed an adjusted multiple-tubes approach (Taberlet et al., 1996; Adams and Waits, 2007) with requirements of a minimum of three positive amplifications for homozygotes and two for heterozygotes. The complete dataset of individual genotypes consisted of 20 microsatellite loci plus amelogenin. Genotypes with more than three missing loci were discarded.

To assess the reliability of individual genotypes we followed the method of Miquel et al. (2006) and calculated a quality index (QI) for each sample. Genotyping errors, i.e., allelic dropout (ADO) and false alleles (FA), were quantified according to Broquet and Petit (2004). The probability of identity among genotypes (P_{ID}; Paetkau and Strobeck, 1994) and the probability of identity considering the possible presence of closely related individuals ($P_{(ID)sib}$; Waits et al., 2001) was calculated in Gimlet v.1.3.3. (Valiére, 2002).

Deviations from Hardy–Weinberg equilibrium (HWE) and presence of linkage disequilibrium (LD) were estimated with the R v.4.0.2 software (R Core Team, 2020) *genepop* package v.1.1.7 (Rousset, 2008) using the following Markov chain parameters: 10,000 dememorization, 500 batches, and 10,000 iterations per batch. The deviations were estimated for each of the four temporal subpopulations (see below) and statistical significance was adjusted according to Holm–Bonferroni method for multiple tests (Holm, 1979) for p-value corresponding to $\alpha = 0.05$. The frequency of null alleles was assessed with Brookfield (1996)'s method as implemented in the *PopGenReport* package v.3.0.4 (Adamack and Gruber, 2014).

2.3. Genetic diversity measures

For genetic diversity analyses, all individual genotypes identified were divided into four groups (*temporal subpopulations*) according to the date of sample collection - BBA A: lynx years 1984–1999, BBA B: 2000–2009, BBA C: 2010–2014, and BBA D: 2015–2019 (Fig. 1). Genetic diversity indices, such as the mean number of alleles per locus (N_A), number of effective alleles (n_e) and private alleles (P_A), observed heterozygosity (H_o), and unbiased expected heterozygosity (H_e) were calculated in Microsatellite Toolkit software (Park, 2001). Allelic richness (AR) was computed using the rarefaction method with correction of variation in sample size in the R package *PopGenReport* v.3.0.4. The inbreeding coefficient F_{IS} was estimated using GENETIX v.4.05.2 (Belkhir et al., 1996–2004), applying 10,000 permutations to assess statistical significance. For evaluation of genetic diversity and structure of BBA in the European context, we used a reference dataset of 98 unique individual genotypes (Table S9, Table S10) previously analysed employing the same set of 15 microsatellite loci for individual identification (Krojerová-Prokešová et al., 2019; Gajdárová et al., 2021): the native Carpathian (source population, N = 42, samples from the lynx distribution range in the Western Carpathians - the Czech Republic and Slovakia, covering 2014–2020), Scandinavian (N = 19), Baltic (N = 14), and reintroduced Harz population (N = 23). All individuals from these reference populations were genotyped for the additional set of five loci for the purpose of this study to allow reliable comparison with BBA. To test the null hypothesis of no change in diversity over time, differences in allelic richness and expected heterozygosity were compared among all BBA temporal subpopulations and the source Carpathian population using Wilcoxon rank-sum test with corrections for multiple testing. Temporal trends in genetic diversity were analysed in detail using a sliding window approach according to Sindičić et al. (2013). The sliding window of 20 individual genotypes was moved forward in time by one individual at each step in the dataset where all individuals were sorted by the date of sample collection. Individuals with samples across several lynx years were treated once in each lynx year. The final dataset for sliding window genetic diversity measures consisted of 131 groups of individual genotypes.

2.4. Analysis of genetic differentiation and population structure

The extent of genetic differentiation among temporal BBA subpopulations and other European lynx populations was quantified by pairwise F_{ST} based on Weir and Cockerham (1984) in the R package *hierfstat* (Goudet, 2005) with 97.5% confidence intervals estimated using 999 bootstrap replicates. A Bayesian clustering procedure implemented in STRUCTURE v.2.3.4 (Pritchard et al., 2000) was run

with 10 independent simulations for each value of K from 1 to 10, with 1 000 000 permutations and an initial burn-in of 100 000 generations. In all simulations, an admixture ancestry model without using sampling locations as prior information and a correlated allele frequency model were used. The K value was estimated by Evanno's calculation (Evanno et al., 2005), which is based on the second order rate of change in the log probability of the data between successive values of K (ΔK), and by estimators accounting for uneven sampling and hierarchical structure (Puechmaille, 2016), both evaluated using the online application StructureSelector (Li and Liu, 2018). The results of independent runs for each K were combined and displayed graphically using the same online application, integrating CLUMPAK (Kopelman et al., 2015).

As an alternative approach, a factorial correspondence analysis (FCA) using GENETIX v.4.05.2 (Belkhir et al., 1996–2004) was performed. This analysis aimed to show the temporal changes in the genetic differentiation of the reintroduced BBA from the source population, so only individual genotypes from the four BBA temporal subpopulations and the native Carpathian population were included.

2.5. Estimation of individual inbreeding and relationships

Individual inbreeding (*F*) was estimated in COANCESTRY v1.0.1.2 (Wang, 2011) implementing a dyadic likelihood estimator (DyadML; Milligan, 2003). The method was chosen instead of other estimators on the base of simulation results (Fig. S1, Table S2) as described in Taylor (2015). For simulations, we used the observed allelic frequencies at the marker set for the most recent temporal BBA subpopulation because of the largest sample size. The missing data and error rates were applied according to the rates estimated in this study (Table 1). Subsequently, 100 pairs of genotypes for different inbreeding categories were generated, including two-selfed sibs, full sibs from one, and two generations of full-sib mating (probability values of nine condensed identity by descent (IBD) configurations were taken from Hedrick and Lacy, 2015). The best estimator was then chosen from a matrix of correlation coefficients among different estimators and the true simulated values (Table S2). *F* estimates were conducted separately for the four temporal BBA subpopulations and the Carpathian population as it was shown that population substructuring can upwardly bias calculations of individual inbreeding (Zilko et al., 2020). Temporal trends in inbreeding were analysed in detail using a sliding window approach as described above. Maximum likelihood estimates of different relationship categories were obtained using ML-RELATE (Kalinowski et al., 2006) and visualised in a social network using the software package Gephi v0.9.2 (Bastian et al., 2009). The information content of each locus and the overall power of the microsatellite marker set for relationship estimation were calculated using KinInfor v2.0 (Wang, 2006).

2.6. Calculations of effective population size

To quantify the rate of genetic drift and inbreeding in BBA we calculated the effective population size using the "single-sample" unbiased linkage disequilibrium (LDNe) estimator (Waples, 2006) as well as the temporal "two-sample" estimator (Jorde and Ryman, 2007), both implemented in the NeEstimator program (Do et al., 2014). For the LDNe approach, we excluded rare alleles according to the formula "$1/(2\,S) < P_{crit} < 1/S$", where P_{crit} is the minimum frequency for alleles to be included in the analysis and S equals sample size. The LDNe estimator has a good precision even for small populations (Waples and Do, 2010), and when the appropriate critical threshold for the lowest allele frequency is applied, it should not be sensitive to sample size (Kamath et al., 2015). Nevertheless, N_e estimates for temporal subpopulations BBA A, B, and C have to be treated with caution due to very small sample sizes (< 25 individuals) and we thus focused on the most recent period with the largest sample size (N = 89) as the most precise estimate for conservation management purposes. The temporal method of Jorde and Ryman (2007) was chosen because it accommodates populations with overlapping generations. As the temporal method is more precise for samples taken more generations apart, when the drift signal is stronger (Waples and Yokota, 2007), we used only the oldest individual genotypes till 1999 (BBA A = generation 0) and the most recent individual genotypes from the last season 2019 (generation t) as the two subsamples being approximately 7.7 generations apart (estimated generation time of the Eurasian lynx for BBA is 2.6 years; Palmero et al., 2021). Result from the temporal method is then corresponding to the harmonic mean N_e in the sampling interval between generation 0 and generation t-1 (Waples, 2005). The program was run assuming random mating and rare alleles with frequencies below 0.05 or below 0.01 (according to the sample sizes) were excluded. The 95% confidence intervals were derived using the "parametric" option (Waples and Do, 2010).

3. Results

3.1. Microsatellite genotyping of collected samples

Microsatellite genotyping was successful in 226 out of 582 samples (38.8%, Table S3) with relatively low average allelic dropout (11.9%) and false allele rates (0.9%) per locus (Table 1). Lower genotyping success (Table S3) was associated with a predominance of non-invasively collected hair samples in which it is difficult to determine their age and there is a higher risk of contamination among individuals, especially if they are collected at marking sites as in this study. The mean quality index across all loci and samples was relatively high (0.88; Table 1), with the lowest quality index of a sample used for statistical analyses being 0.44. The final dataset of individuals consisted of 121 unique multi-locus genotypes (≤ 3 missing loci out of 20 loci allowed). After Holm–Bonferroni correction for multiple testing, no HWE deviation was confirmed at any locus. One locus was monomorphic in BBA, but we decided not to discard it in subsequent analyses as it is polymorphic in other populations included in this study. Null alleles were not observed at any locus. Linkage disequilibrium (LD) was observed after Holm–Bonferroni correction in two pairs of loci (F53/FCA096 and FCA742/FCA650), but we decided to not discard them as LD at neutral loci in isolated populations might be caused by genetic drift and so it can be used

for effective population size estimation (Hill, 1981). The power of the panel of first 15 microsatellite loci (used for individual identification) to distinguish between closely related individuals was high ($P_{(ID)sib} = 3.31 \times 10^{-4}$), for the entire panel of 20 loci, the power of the panel increased further ($P_{(ID)sib} = 9.63 \times 10^{-6}$).

3.2. Genetic diversity

We identified 121 unique individual BBA genotypes out of 226 successfully genotyped samples (Table S9). Four individuals were detected in two successive temporal subpopulations (see Methods), so their genotypes were included in both. One male sampled close to BBA area clearly assigned to the Harz population (see below) and was excluded from analyses of genetic diversity of BBA. We found a statistically significant decrease (Wilcoxon rank-sum test, $p \leq 0.05$, Table S4) in both AR and H_e only in the most recent temporal subpopulation BBA D in comparison to the oldest BBA A subpopulation. For H_e there was also a decrease in BBA C in comparison to BBA A subpopulation. No significant differences in the genetic diversity were found in the last two decades (2000–2019) in pairwise comparison between BBA B, C, and D (Fig. 2). BBA A was the only temporal subpopulation with levels of genetic diversity similar to the source Carpathian population. Seven alleles were found only in BBA A, and they were most likely lost in the first years after

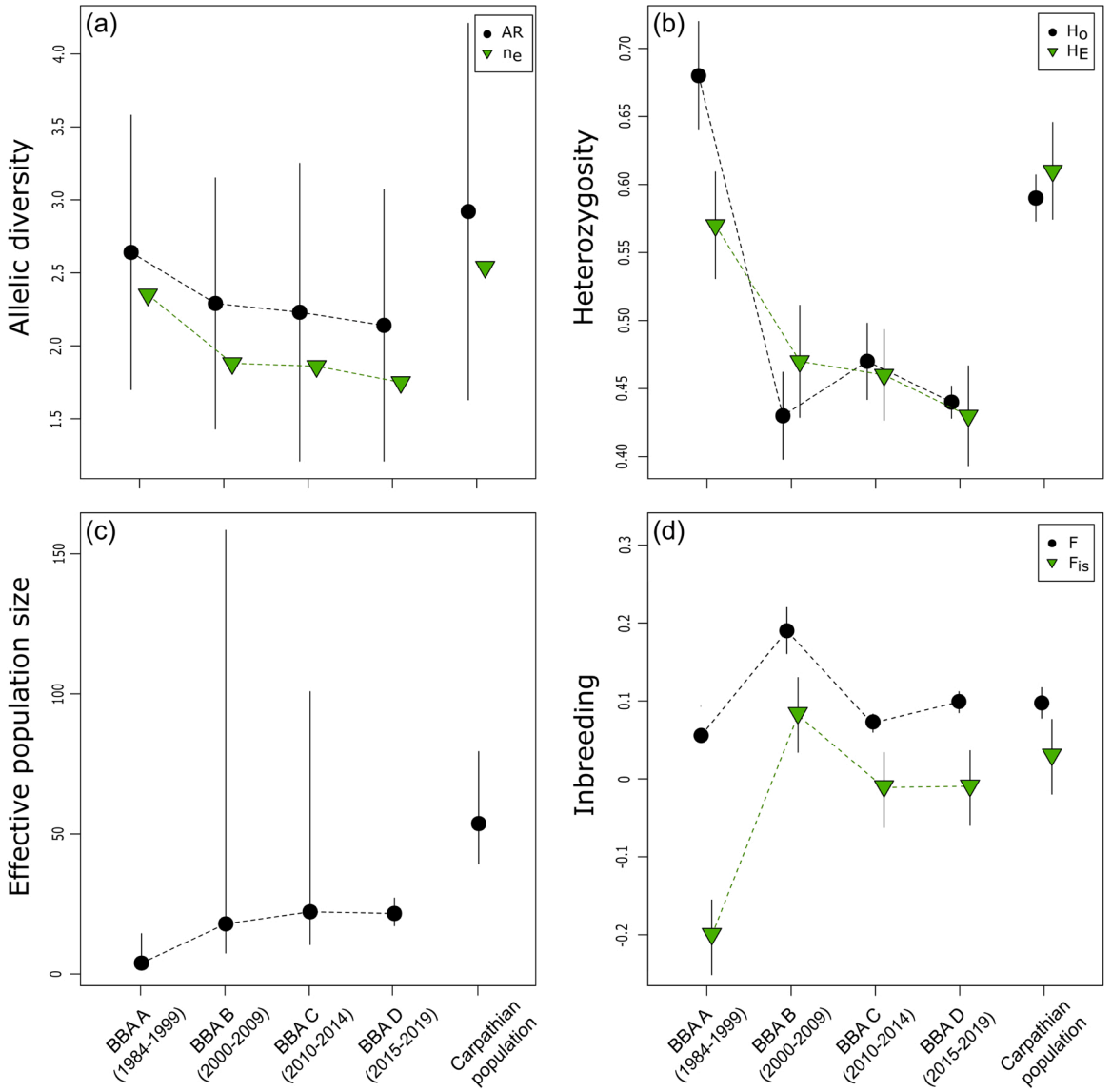

Fig. 2. Temporal trends in BBA for (a) the allelic diversity (n_e – effective number of alleles, AR – allelic richness with standard deviation), (b) observed (H_o) and expected heterozygosity (H_e) with standard deviations, (c) effective population size with 95% CI (LDNe method), (d) average individual inbreeding *F* with variance and Wright's inbreeding coefficient F_{IS} with 95% CI. The values for the source Carpathian population are shown for comparison.

reintroduction. Three alleles appeared only in BBA B or C, but with low frequency, and they were not present in the most recent one - BBA D. One allele was private for BBA (detected in BBA A and D); however, the allele has been detected at a low frequency in older samples from the Carpathians (Krojerová-Prokešová J., unpublished data). All other alleles detected in BBA were observed also in the source Carpathian population. Genetic diversity values for BBA and other European populations are shown in Table 2. The sliding window analysis showed a considerable decrease in genetic diversity during the period A, which continued much more slowly in the following periods. Moreover, in the second half of the most recent BBA D period, a slight increase was noticeable (Fig. 3).

3.3. Genetic differentiation between BBA and adjacent populations

The pairwise F_{ST} values confirmed significant differentiation between all European lynx populations included in this study (Table S6). No genetic difference was found between temporally adjacent BBA subpopulations (BBA A vs. B, B vs. C, and C vs. D), although differences were apparent between populations with greater temporal separation (F_{ST} = 0.03–0.08). In the same context, differentiation between BBA and the source Carpathian population increased over time from F_{ST} = 0.03 (BBA A vs. Carpathian) to F_{ST} = 0.14 in the most recent period (Table S6).

Bayesian clustering showed that the oldest historical samples from the period BBA A clearly clustered with the source Carpathian population. However, during first 15 years after reintroduction, BBA differentiated from the source Carpathian population as well as from other studied European lynx populations and has formed a separate genetic cluster (Fig. 4; see more details about Bayesian clustering in Appendix 1). The gradual process of genetic drift of BBA from its source Carpathian population is visible also using FCA (Fig. 5). One individual adjacent to BBA clearly related to the Harz population (Fig. 4). No other long-distance dispersers from adjacent populations were identified within and/or in the vicinity of BBA in this study.

3.4. Inbreeding and individual relationships

According to simulations in COANCESTRY, the DyadML method provided the highest precision and together with the TrioML method also the lowest bias (Table S2). Even though the estimates of F for individuals ranged widely, the mean values derived for temporal subpopulations were more reliable being very close to simulated true values of inbreeding coefficients (Fig. S1). The average inbreeding coefficient was low within the group of founders and their direct offspring (BBA A) (0.055; Fig. 2). In the second temporal subpopulation, the value was the highest (0.190), which indicates frequent breeding between relatives. Inbreeding subsequently dropped in the growing population and in the most recent period, reached a value (F = 0.098) similar to that of the Carpathian population (F = 0.097), indicating only low inbreeding, even though the actual level should be treated with caution (see more in Discussion). Individual inbreeding and F_{IS} analysed using sliding window approach reached two peaks, the first one at the end of period A and the second one in the middle of period D, after that the values again decreased (Fig. 3).

The accuracy for distinguishing full-siblings from unrelated pairs was 86%, 79% for full-siblings (FS) versus parent-offspring pairs (PO) (Table S7), thus we aimed to distinguish only between first-degree relationships (FS together with PO) against unrelated pairs. These first-degree relationships were visualised in georeferenced social networks, indicating no severe geographical barriers for gene flow within BBA (Fig. 6).

3.5. Effective population size

The average N_e over an unspecified number of previous generations calculated using the LDNe method was low in years shortly after the reintroduction (BBA A: N_e = 3.9, 95% CI = 2.1–14.4), but then increased and remained at similar level of approximately N_e = 20 in each of the following periods (Fig. 2, Table S8). However, as indicated by the narrowest confidence interval, the LDNe method probably yielded the most precise estimate for the most recent time period with the largest sample size (BBA D: N_e = 21.6, 95% CI = 17.2–27.1; Table S8). The effective population size was estimated to be higher using the temporal method, N_e = 35.6, 95% CI = 22.4–51.7; Table S8). Using the estimate of 121 (19% standard deviation: 98–144) independent individuals (Mináriková et al., 2019) what is closest to the period corresponding to effective population size calculation, the overall effective population size to adult census size ratio (N_e/N) was 0.179 (0.176–0.188) and 0.294 (0.229–0.359) using the LDNe and temporal methods, respectively.

Table 2
Genetic diversity estimates for BBA, divided into four time periods, and for other European populations. N = number of individuals, N_A = number of alleles, n_e = number of effective alleles, AR = allelic richness, H_o = observed heterozygosity, H_e = expected heterozygosity, F_{is} = inbreeding coefficient.

Population	N	N_A	n_e	AR	H_o	H_e	F_{is}
BBA A (1984–99)	7	3.05	2.35	2.64	0.68	0.57	-0.203
BBA B (2000–09)	12	2.70	1.88	2.29	0.43	0.47	0.084
BBA C (2010–14)	16	2.75	1.86	2.23	0.47	0.46	-0.027
BBA D (2015–19)	89	2.85	1.75	2.14	0.44	0.43	-0.025
Carpathian	42	4.25	2.54	2.92	0.59	0.61	0.031
Scandinavian	19	3.90	2.00	2.63	0.50	0.50	0.004
Baltic	14	4.90	2.86	3.32	0.60	0.65	0.086
Harz	23	3.65	2.20	2.71	0.55	0.54	-0.006

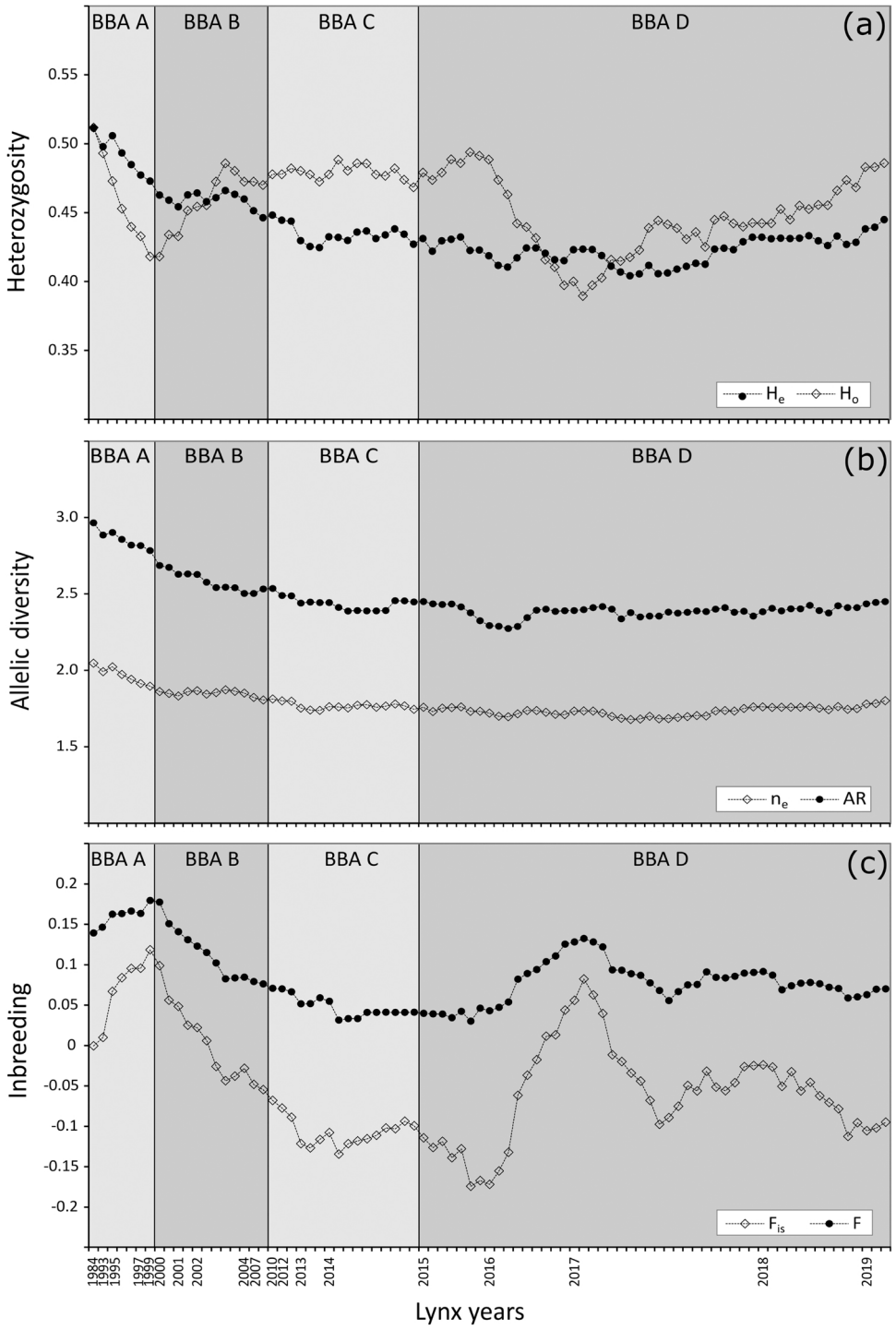

Fig. 3. Temporal trends in genetic diversity and inbreeding in BBA analysed using a sliding window approach (20 individuals in each group of genotypes): (a) expected (H_e) and observed (H_o) heterozygosity; (b) allelic richness (AR) and the effective number of alleles (n_e); (c) average individual inbreeding (F) and Wright's inbreeding coefficient (F_{is}). Within the most recent period D, the number of groups was reduced by every second group due to the large sample size in lynx years 2016, 2017, and 2018. Number of analysed samples and individual genotypes for lynx years in BBA D temporal subpopulation are given in Table S5.

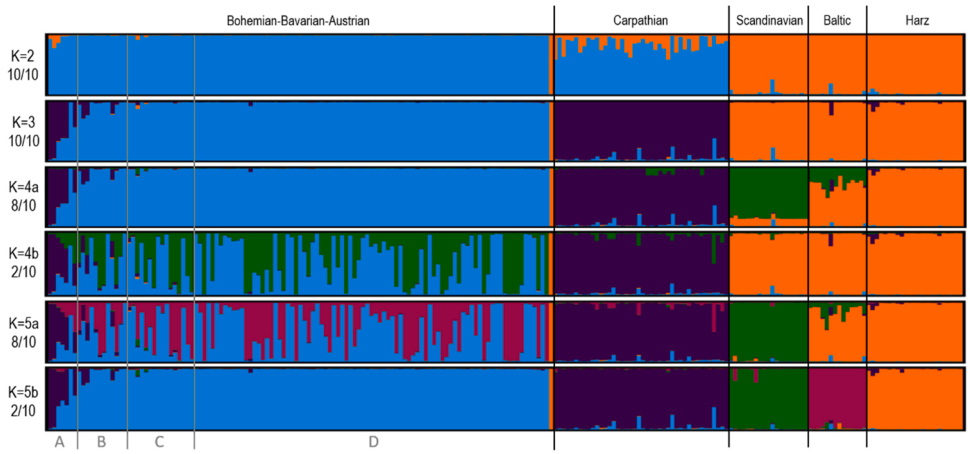

Fig. 4. Microsatellite-based genetic sub-structuring of five European lynx populations using Bayesian clustering in software STRUCTURE for K= 2–5. Each column represents one animal, the colour of each column corresponds to the probability of the assignment to a certain cluster. A, B, C and D on X-axis indicates four BBA temporal subpopulations. For K = 4 and 5 major (a) and minor (b) clusters are shown as identified by Clumpak. The frequency of these clusters among 10 runs is given under the K-value.

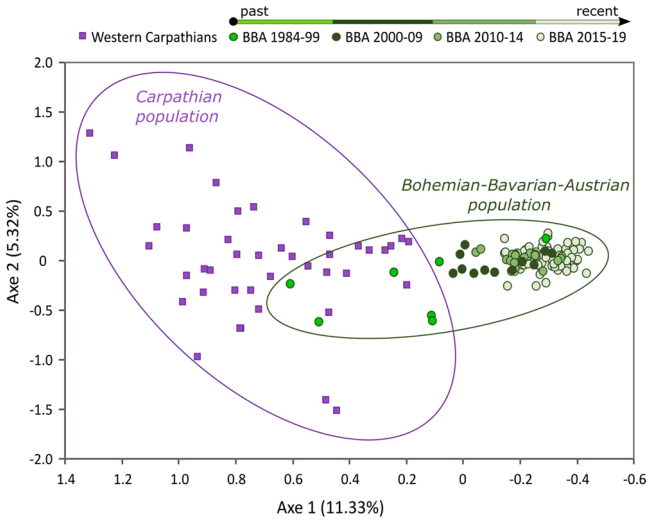

Fig. 5. Factorial correspondence analysis showing genetic differentiation between BBA and its Carpathian source population over time.

4. Discussion

4.1. Genetic diversity over 35 years

Using the whole dataset of past and recent lynx samples we found that BBA lost about 25% of expected heterozygosity and 19% of allelic richness over the whole study period (1984–2019). A similar decline in genetic diversity (heterozygosity and/or allelic richness) was detected in a bottlenecked population of African lions (12–17%; *Panthera leo*; Dures et al., 2019). However, the grade of genetic diversity loss is somewhat less severe compared to values found in several other endangered species, e.g., 30–40% in grey wolf in Scandinavia (*Canis lupus*; Flagstad et al., 2003), 57% in the Mauritius kestrel (*Falco punctatus*; Groombridge et al., 2000), or 43% in sea otter (*Enhydra lutris*; Larson et al., 2002).

Most of the decline is, however, connected with the period soon after reintroduction. Interestingly, the observed heterozygosity in this first period was higher than expected and even higher than in other native European lynx populations (Table 2, Fig. 2). This could be explained by two waves of reintroduction in 1970s and 1980s and by an isolate-breaking effect (Hamrick et al., 1991), when individuals from at least two partially isolated mountain ranges of the Western Carpathians were released (Volfová and Toman, 2018). This build-up of increased levels of genetic diversity following multiple reintroductions and presumed subsequent rapid population growth (at the end of 90s the population size was estimated to be 100–150 individuals; Anděra and Gaisler, 2012) could have helped to slow down the decline in genetic diversity (*Allee effect*). Our results correspond to theoretical considerations that in populations

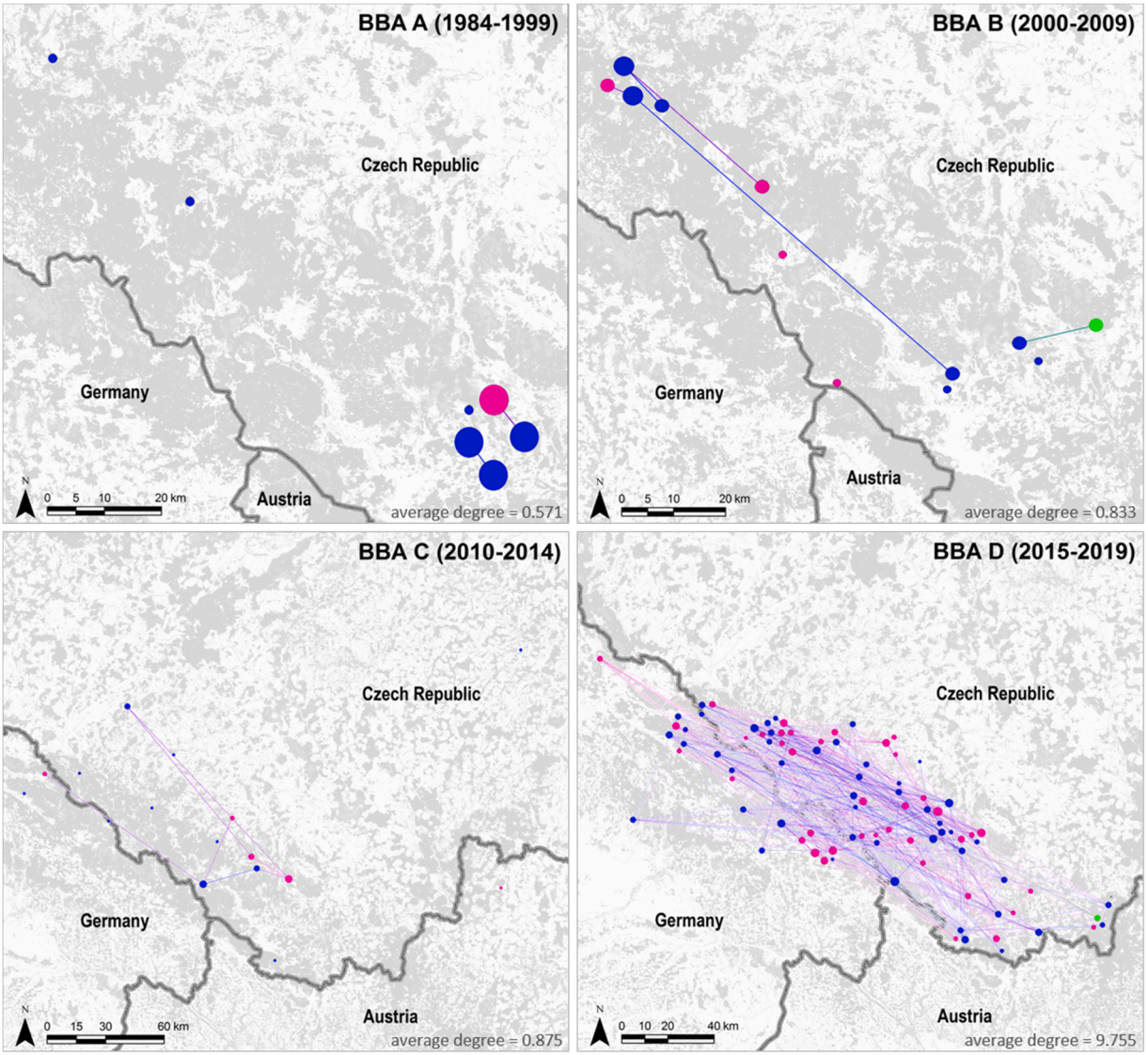

Fig. 6. First degree relationships (parent-offspring, full sibs) between individuals in the four BBA temporal subpopulations. Each node represents one individual, males = blue, females = pink, unknown sex = green colour. Nodes are georeferenced (their position refers to geographical coordinates of the sample), with a function of no overlap. The size of nodes corresponds relatively to the average number of relationships per node (average degree) computed separately for each period. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

showing rapid population growth after going through a bottleneck, the loss of heterozygosity occurs mainly in early generations, reaching a stable level of heterozygosity at a certain degree of population growth (Nei et al., 1975).

After this initial phase of rapid population growth, the population size of BBA decreased (the estimate for this period was around 50 individuals; Kaczensky et al., 2013) and closely related descendants were thus more likely to mate with each other, which probably resulted in lower H_o in the subsequent period, indicated also by increased inbreeding. Assessment of fine-scale temporal changes using a sliding window approach confirmed a slight decrease in H_o and an increase in inbreeding not only for BBA A but also in the middle of the period D (lynx years 2016 and 2017). The latter could be explained by stochasticity in sampling, when in the lynx year 2017 we detected almost double number of individuals comparing to other lynx years in the period D (Table S5), but one quarter of them were dead kittens from few litters. However, AR and H_e remained stable during this second fluctuation contrary to the changes in all parameters detected previously for BBA A, which suggests AR and H_e are better indicators of real long-term as well as short-term changes in genetic diversity. In addition, there has been a slight increase of these parameters over the last period (2015–2019) as evidenced from the sliding window analysis, which is unlikely explained by a bigger sample size in last years (Table S5). Rather, it corresponded to a documented increase in population size (Mináriková et al., 2019; Wölfl et al., 2020; Palmero et al., 2021).

Given their large home range sizes combined with low population densities, large carnivores generally exhibit lower levels of

genetic variation than other mammals (Azizan and Paradis, 2021; Merola, 1994). In European lynx populations, this pattern was exacerbated over its past and recent evolutionary history due to climatic oscillations and recent anthropogenic pressure (Lucena-Perez et al., 2020). In the Carpathian lynx population genetic diversity has been particularly depleted due to isolation during the last glacial maximum (the Carpathians served as a glacial refugium for lynx; Sommer and Nadachowski, 2006) as well as a strong bottleneck in 1930s due to human persecution in the West Carpathians (Hell, 1968; Jamnicky, 1997). Thus, the observed decline in genetic diversity of BBA, founded by individuals from the Western Carpathians, is unlikely to be as intensive as in other endangered species discussed above, as the population has already been depleted prior to reintroduction.

Moreover, genetic diversity is usually less reduced through time in iteroparous species (Johnson, 1977; Kaeuffer et al., 2007; Kekkonen et al., 2012) and generally higher in species with shorter generation time because this determines the speed of evolutionary response to selection (Araya-Ajoy et al., 2021). The generation time calculated for BBA is shorter (2.6 years, Palmero et al., 2021) than the estimate previously reported for other lynx populations (4 years; Spong and Hellborg, 2002). If adult mortality is very high, then natural selection favours individuals that allocate energy towards reproduction earlier in life and contrary, high juvenile mortality favours individuals that allocate more in self-maintenance so that they have the chance to reproduce in several breeding seasons (Michod, 1979; Araya-Ajoy et al., 2021). The shorter generation time observed in BBA might be a consequence of high adult mortality, most probably caused by poaching (Červený et al., 2019), but it can also act as defence mechanism helping to maintain genetic diversity and population growth. Shorter generation time and larger population size are supposed to be important demographic contributors to maintain neutral genetic diversity (Hague and Routman, 2016) and both probably also affected genetic diversity of BBA.

4.2. Population isolation

Unlike Bull et al. (2016), who identified one lynx in BBA that appeared to be admixed with other lynx populations (although it must be noted, that the admixed lynx could also simply stem from an enclosure escape), we did not find any individuals of different or admixed origin in BBA. Moreover, we did not detect any alleles in BBA, which do not occur in the Carpathians except for a single private allele of unknown origin. We thus found no signs of allelic enrichment due to immigration from other populations, and a lack of past or recent admixture. One possible immigrant was identified as an individual belonging to the Harz population, but the sampling site (Friedenfels, Bavaria) was 70.5 km straight line distance to the closest EEA square with the permanent occurrence of BBA (Fig. 1). BBA thus still seems to be isolated from all neighbouring lynx populations, even though few other dispersers have been recorded halfway between BBA and the Carpathian or the Harz population (Gajdárová et al., 2021).

4.3. Inbreeding estimates

Estimation of F at an individual level is problematic for threatened species with small population sizes and low genetic diversity and the estimates are relative to a reference population used in calculations (Wang, 2014; Taylor, 2015). The inbreeding estimates thus should be applied mainly for assessing the temporal changes or for comparison of average values among populations rather than for evaluation of actual level of inbreeding of particular individuals, which was confirmed also by simulations done in this study (Fig. S1).

The low level of BBA inbreeding in the early phase after reintroduction indicates that mostly unrelated individuals were released. However, because of the small founder size (even though being among the highest in comparison to other reintroduced lynx populations; see Mueller et al., 2022), breeding in a small group ensued in mating between relatives, leading to increased inbreeding in the following BBA B period. Surprisingly, in the two most recent time periods we found no substantial inbreeding, probably due to increasing population size (Mináriková et al., 2019; Wölfl et al., 2020).

Further, it has been described that lynx, despite being a solitary carnivore, can form kin clusters (Holmala et al., 2018; Krojerová-Prokešová et al., 2019) and do not avoid mating between relatives, especially in areas with some degree of landscape fragmentation (Krojerová-Prokešová et al., 2019). However, no such kin clusters were found in the case of BBA. The absence of fragmentation of the Bohemian Forest Ecosystem allows the population to function as one compact cluster and hence also to reduce inbreeding at the population level.

As a result, the most recent inbreeding estimate for BBA is similar to that in the Carpathian source population, in which an increased level of inbreeding in comparison to other native European lynx populations has been highlighted recently in a genome-wide assessment (Mueller et al., 2022) as a result of several bottlenecks during its history. At the same time, however, it cannot be excluded that these bottlenecks could increase purging in this population (and in all reintroduced populations of Carpathian origin), which may result in a partial reduction of deleterious mutation load, making these populations more resilient to subsequent bottleneck events (Kleinman-Ruiz et al., 2022).

4.4. Effective population size

The final aim of this study was to estimate the contemporary effective population size of BBA representing one of the main parameters for conservation management as it quantifies the magnitude of genetic changes (Ryman et al., 2019; Wang et al., 2016). Result of the LDNe method in the period closest to the establishment of the population ($N_e = 3.9$) implied that the real number of founders might be probably much lower than the number of released individuals (N = 22–24, Červený and Bufka, 1996, Volfová and Toman, 2018), although the N_e could be biased by small sample size. Two decades after the release, N_e has increased and remained at almost the same level for at least the next 20 years, with the most relevant estimate for conservation management purposes being $N_e = 21.6$ (17.2–27.1). This is in contrast for example to the Dinaric lynx population, in which the effective population size has dropped

from $N_e = 35.9$ to only 13.4 over the same time span (Skrbinšek et al., 2019), although this population is currently undergoing an extensive genetic recovery program aiming to improve the low genetic diversity by translocation of lynx from the Carpathians. In the Iberian lynx population with $N_e < 25$, evidence of inbreeding depression has been documented (Casas-Marce et al., 2013), indicating that similar problems may occur in BBA in future.

The temporal N_e estimate (N_e =35.6, 95% CI = 22.4–51.7) was slightly higher than the estimate of the LDNe method, but as it was shown in other studies, the time period between the two temporal subsamples should be in practice even longer to produce unbiased estimates, especially in iteroparous mammals with overlapping generations (Waples and Yokota, 2007; Skrbinšek et al., 2019). In our case, the first subsample (1984–1999) probably spans more than one generation which could further bias the temporal N_e estimate.

Both N_e estimations are, however, below the recommendations of Frankham et al. (2014) of $N_e \geq 100$ for inbreeding depression avoidance over five generations, even though there has been an ongoing debate about the value (García-Dorado, 2015; García-Dorado and Caballero, 2021) and its generalisation across species and taxa (Wang et al., 2019). Recent genomic analyses have shown that populations with an effective size below 100 individuals can accumulate a substantial burden of mildly deleterious mutations, associated with higher long-term extinction risks, although highly deleterious mutations responsible for short-term extinction are purged (Grossen et al., 2020). However, also populations of sizes of ~1000 still accumulate mildly deleterious mutations (Grossen et al., 2020). Due to habitat restrictions, it is unrealistic to increase the size of BBA or all other reintroduced lynx populations in Europe above this threshold, making long-term conservation management of this species challenging. The concept of a European lynx metapopulation facilitating gene flow between neighbouring populations appears to be the only sustainable option (Bonn Expert Group, 2021).

5. Conclusions

Our results revealed that BBA is genetically impoverished in comparison to native European lynx populations, with effective population size estimated at about 22 individuals, which is far below the recommended level to prevent inbreeding and avoid further loss of genetic diversity. Due to genetic drift and isolation, BBA has undergone rapid genetic differentiation from the source Carpathian population within the first decade since reintroduction. However, a two-wave reintroduction of individuals from at least two distinct areas in the West Carpathians resulted in relatively high start-up of genetic diversity, which together with population growth during 1990s led to the maintenance of genetic diversity and effective population size over the last 20 years (1999–2019). Moreover, recent multi-season camera-trapping data showed that the population is slightly increasing (Mináriková et al., 2019; Wölfl et al., 2020; Palmero et al., 2021). Even though our results support the supposed isolation of BBA and thus absence of gene flow, we also detected relatively low inbreeding during two recent decades that probably helped to prevent further significant reduction of genetic diversity. The full protection of the population since its establishment together with sufficient food supply and compact permeable habitat probably helped to achieve the population recovery and growth in contrast to that of the Dinaric or Alpine populations, in which over-hunting together with illegal killing broke the previous population expansion and contributed to the population decline (Molinari-Jobin et al., 2003), in the Alps further exacerbated by habitat fragmentation (Kaczensky et al., 2013). Our results highlighted the need to study populations separately and to analyse their temporal genetic changes in order to more accurately determine population conservation status and subsequent management.

6. Conservation implications

On the base of our results which do not indicate the ongoing loss of genetic diversity and together with the fact that recent demographic data reported slight population increase, the need of genetic rescue as a suitable conservation management measure to preserve the evolutionary potential of BBA currently seems to be questionable. Genetic rescue through further reintroductions should be attempted when a population shows signs of inbreeding depression such as negative population growth without any apparent environmental cause, high susceptibility to pathogens, poor physical condition of individuals, occurrence of morphological deformities, or lowered fecundity and survival compared with larger populations in similar environments (Reed, 2010). No such signs have been detected in BBA up to date. Further, genetic rescue should be considered only when there is a prospect for a rapid growth of a rescued population, primary factors threatening population are removed and when it is clear the benefits outweigh the risks (Hedrick and Fredrickson, 2010; Kleinman-Ruiz et al., 2022). Any human-mediated translocations bring with them several challenges that need to be considered and balanced against the potential benefits, e.g., resistance of local hunters resulting in increased rates of illegal killing (Červený et al., 2019), or the lack of suitable individuals used for the reinforcement (the favourable status of the source Carpathian population is questionable; Krojerová-Prokešová et al., 2019, Kubala et al., 2020, Mueller et al., 2022, von Arx, 2020, Bonn Expert Group, 2021). Moreover, the impact of genetic rescue on the long-term viability of a population is under debate (Hedrick and García-Dorado, 2016), and in special cases it could be an even harmful strategy, as animals translocated from a larger source population may introduce new deleterious mutations (see example of the Isle Royal wolf population), which have been purged in the smaller donor population (Hedrick and García-Dorado, 2016; Robinson et al., 2018; Hedrick et al., 2019; Kleinman-Ruiz et al., 2022). Furthermore, in the light of the most recent genomic study of the Iberian and Eurasian lynx (Kleinman-Ruiz et al., 2022), it has been argued that the improvement of landscape connectivity necessary for the restoration of natural gene flow between small, likely purged populations should be preferred over translocations from larger populations.

The strength at which evolutionary processes influence levels of genetic diversity is dependent on the long-term effective population size. However, evolutionary forces are not a sole driver and interact with biological and human-mediated factors (Sonsthagen et al., 2017), together influencing the maintenance and recovery of genetic diversity after a bottleneck event. It is indisputable that

BBA is vulnerable with low effective population size, thus it is essential to maintain the current positive trend of population growth and to prevent future decline due to human induced mortality (illegal killing, vehicle collisions) as the main driver limiting a substantial population growth and hindering dispersal to surrounding suitable habitats (Červený et al., 2019; Mináriková et al., 2019; Wölfl et al., 2020).

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CRediT authorship contribution statement

BG and JKP performed laboratory analyses and designed the study. BG and JKP wrote the first draft of the manuscript and performed the statistical analyses. All authors collected samples and/or edited/approved previous versions of the 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.

Data availability

The genotype data are available within the supplementary material of this article.

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Animal research

Genetic material was collected following institutional, national, and international guidelines. No animals were killed due to sampling and therefore a formal approval by an Institutional Ethical Committee was not necessary.

Consent to participate

The manuscript does not contain any individual person's data – not applicable.

Consent to publish

All authors consent to submitting this article to Global Ecology and Conservation.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2023.e02399.

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