Preventing the extinction of the Dinaric-SE Alpine lynx population through reinforcement and long-term conservation

LIFE16 NAT/SI/000634

Baseline (pre-reinforcement) genetic status of SE Alpine and Dinaric Lynx population

Action A3

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September 2019

Summary

Dinaric lynx population, which went extinct at the turn of the 20th century, was reintroduced in 1973. The reintroduction was done with three pairs of lynx translocated from Slovak Carpathians to an area close to Kočevje in SE Slovenia. While the population initially did very well and expanded both numerically and spatially, we have been observing its decline over the last two decades. Considering genetic erosion as one of the main causes, the project LIFE Lynx was started to counter its effects and prevent the population extinction. However, as it is impossible to reach the critical population viability thresholds within a single LIFE project, we intend to make a long-term plan for genetic management of the population as one of the concluding actions of the project. To be able to do that, we must have an understanding how the genetic picture developed after the 1973 reintroduction, and what is the "baseline" status of the population before the population augmentation that started in 2019. That was the main goal of this project action, and its genetic component is the main topic of this report.

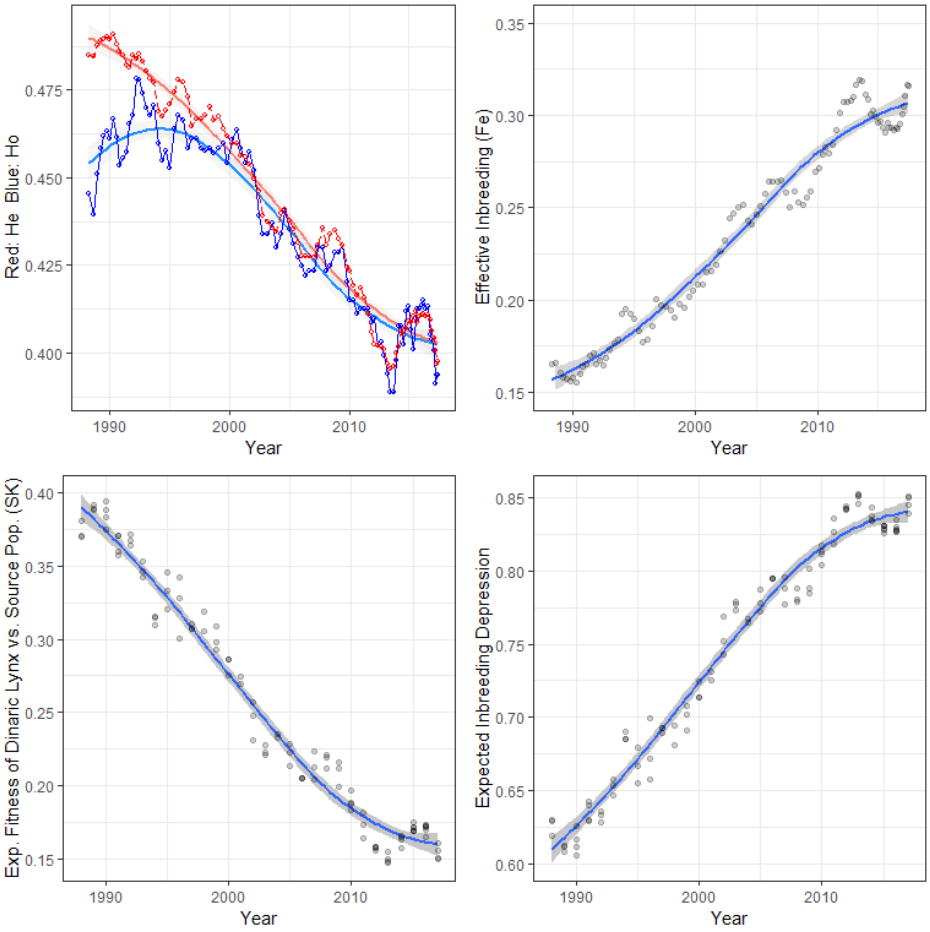

Figure I: Heterozygosity (upper left), effective inbreeding (upper right), expected inbreeding depression (lower right) and expected remaining fitness of the Dinaric lynx population (lower left) relative to the source population in Slovak Carpathians. We used 12 diploid lethal equivalents (O'Grady et al. 2006) *to estimate inbreeding depression.*

We included genotypes of 219 animals in this study – 149 from the NW Dinaric Mountains, 48 from Slovakia and 22 from Romania. We could see a clear genetic structure in the Carpathians between Slovakia and Romania, but also a very clear separation of the Dinaric lynx. Compared to the population in Slovakia (*He* = 0.583, *SE* = 0.031) and Romania (*He* = 0.545, *SE* = 0.039), the Dinaric population has considerably lower genetic diversity (*He* = 0.457, *SE* = 0.036) even when the entire time period since the reintroduction is considered.

Although the population did well soon after the 1973 reintroduction, which is indicated also in its relatively high effective population size estimates from that period indicative of population expansion, it lost considerable proportion of genetic diversity already through the reintroduction bottleneck. High genetic drift caused by small effective population size and limited number of unrelated mates immediately after the reintroduction caused rapid inbreeding, followed in-step by the related drop in heterozygosity. While it seems that the population was still doing well in the 1980s when inbreeding coefficient is estimated at *Fe* = 0.176, by the 1990s this parameter reached *Fe* = 0.192. At this level of inbreeding we can already expect 68% drop in fitness (δ = 0.684) at the population level, and this is possibly the time when the first effects of inbreeding depression started. By the 2000s, when field reports started indicating decreasing lynx population, inbreeding already reached *Fe* = 0.26, meaning that on average the Dinaric lynx were more inbred than offspring produced by mating between brothers and sisters. The expected drop in fitness would be 80%.

The pre-reinforcement 'baseline' are the estimates for the last period, 2016 – 2019. Heterozygosity dropped to *He* = 0.399 (*SE* = 0.039), inbreeding reached *F* = 0.316, with the corresponding expected 85% drop in fitness due to inbreeding depression compared to the source population in Slovak Carpathians. The effective population size seems to be at approximately the same level (13.4, 10.2 - 17.5 95% CI) for the last two decades. This is far from the rule-of-the-thumb threshold of the minimum for short-term population viability (*Ne* > 50) that should allow a population to avoid inbreeding depression, and if it stays the same, we can expect 3.7% (2.9 – 4.9%, 95% CI) drop in genetic diversity and increase in inbreeding per generation.

The actual inbreeding depression is hard to determine, but its effects are never negligible in populations where inbreeding has reached the level we're observing here, particularly for the populations in the wild. There may have been some purging of the lethal recessive alleles through selection (which may explain why loss of genetic diversity seems slower in the last decade), but this could have solved only a smaller part of the problem. The deleterious alleles that are under weaker selection and cannot get purged because of the small effective population size still remain.

All in all, the population has not been doing well from the genetic perspective, and as the field data indicate, was going into the "extinction vortex". It's difficult to predict when the population would go extinct without intervention, but there is little doubt that extinction would be a matter of "when" rather than "if". Even though the genetic restoration of the population carries its own caveats, the chances of survival of the Dinaric lynx should improve with each new translocated animal. But even while LIFE Lynx should save the Dinaric lynx population from immediate extinction, it's not the proverbial "silver bullet". The population will need genetic management in the long run to maintain its viability. The experiences obtained through this project will be invaluable in planning such genetic management, and a firm understanding of the "baseline" status immediately prior to population reinforcement that this study provides is the critical first step.

Contents

Introduction

The Dinaric lynx population, which went extinct at the end of the 19th or beginning of the 20th century, was reintroduced in 1973. Six animals were brought from Slovak Carpathians and released in south-eastern Slovenia close to town Kočevje (Čop & Frković 1998; Kos et al. 2004).

The reintroduction was an incredible success, and population rapidly grew and expanded (Čop & Frković 1998; Kos et al. 2004). However, with only six animals as founders, some of them even related, and isolated from all other populations, the lynx in NW Dinaric Mountains soon had no other option than to mate with relatives. Inbreeding started accumulating, and in the 2000s we started observing population decline (Kaczensky et al. 2012). By 2010s signs of lynx presence in the field became increasingly rare, and extinction of the population became a tangible possibility.

Genetic surveys of the Dinaric lynx after 2010 showed that the population had the lowest genetic diversity of all studied lynx populations (Sindičić et al. 2013). The average inbreeding coefficient exceeded 0.25, which is expected in a brother-sister mating. Every sexually reproducing organism carries a load of deleterious recessive or semi-recessive alleles. In a large, outbreeding population these alleles don't do much harm – as each of them is very rare, the probability of an individual obtaining the same deleterious recessive allele from both parents (causing its phenotypic expression) is low. However, in inbred individuals, where both maternal and paternal line meet in a recent ancestor, this probability becomes high, and expression of such alleles causes a decrease in the individual's fitness – lower survival, and lower reproductive success (Allendorf & Luikart 2009). When this is happening at the level of a population, we are talking about inbreeding depression, which can be a major factor in the extinction of a small population.

Our main goal in project LIFE Lynx is to prevent this extinction from happening in the Dinaric lynx population. However, even after the project the population will likely remain small and isolated and will need long-term help to keep it viable in the future. Within the project we have a unique opportunity to closely monitor the population reinforcement, see the actual contributions of the translocated animals and the effect each animal is expected to have on the population's genetic outlook and viability. But, critically, we need a baseline to compare to – the status of the population prior to any new animals being introduced. This was one of the main goals of this project action, and its genetic component is the topic of this report.

Materials and methods

Genetic samples, collection and storage

We used different sources of genetic material:

To obtain historical genetic data, we sampled lynx hunting trophies in Slovenia and Croatia. This was done prior to this project (Polanc et al. 2012; Sindičić et al. 2013), but we used the published data also for the purposes of this study (N=90).

Already before the project we have been using any opportunity to collect lynx samples: tissues from dead lynx, genetic samples of lynx captured for telemetry, and noninvasive samples – scats, urine, hair and saliva found in the field during lynx snow tracking or other activities, or opportunistically sampled during systematic genetic monitoring of wolves and bears (N=32). Within the project, any material that could be used to obtain viable lynx DNA was systematically collected (N=27). This report also includes samples collected in the Slovak (N=48) and Romanian (N=22) part of the Carpathians, the source populations for the planned lynx reintroduction.

Tissue and scat samples were stored in 95% non-denatured ethanol. Urine samples (collected in snow) were stored in DETs buffer, and hair samples were stored in sealed bags with desiccant (silica). Saliva samples were collected with forensic swabs that already have desiccant in the swab tube. While tissue and scat samples are stable and have been kept in storage medium at -20°C until analysis, urine, saliva and hair samples had DNA extracted as soon as possible, with the DNA extract being stored until further analysis.

Laboratory organization and contamination prevention

DNA in noninvasive and historic genetic samples is of very low quality and quantity, and contamination (especially with PCR products) is a serious issue. We used a dedicated laboratory for noninvasive genetic samples for DNA extraction from noninvasive samples and PCR setup (Skrbinšek et al. 2019). The laboratory and an area next to it were also used for storage of consumables and samples. All downstream post-PCR laboratories (PCR, purification of libraries, storage of PCR products) were physically separated on the other side of the building. We enforced strict rules regarding movement of personnel, equipment and material to prevent contamination, and used negative controls throughout. The most basic rule is that any equipment or material that has been to post-PCR areas can never go into the laboratory for noninvasive samples, and personnel that has been to post-PCR areas can only go back in that laboratory when they changed their clothes and have taken a shower.

DNA extraction using laboratory robotics

DNA extraction is a critical part of the genotyping process since it defines the reliability and success of the entire downstream analysis. Noninvasive genetic samples are a difficult material that needs to be handled appropriately. DNA extraction from historic samples is described in Polanc et al. (2012), and manual extraction from noninvasive samples in Skrbinšek et al. (2010, 2019). For contemporary noninvasive samples, we used MagMAX DNA Multi-sample Kit (Thermo Fisher Scientific). The extraction protocol is implemented on a liquid handling robot (Hamilton Starlet) to achieve reliable, error-free and fast DNA extraction. Besides speeding the analyses, use of the liquid handling robot practically eliminated the possibility of a sample mixup since all sample handling is done automatically, and sample IDs read and handled through barcodes. The liquid handling robot is located in the "noninvasive genetics laboratory" and used exclusively for noninvasive and historic samples.

Since their number is manageable, DNA extraction from tissue samples is done using manual DNA extraction kit (Sigma GenElute) in the "tissue laboratory". The procedure is described in Skrbinšek et al. (2012).

Genotyping

We used ten microsatellite markers for individual ID run in a single multiplex: Fca132, Fca201, Fca247, Fca293, Fca391, Fca424, Fca567, Fca650, Fca723, Fca82. SRY locus was used to determine sex of the animal. The best (reference) sample of each detected animal was amplified using 9 additional markers (F115, F53, Fca001, Fca132, Fca161, Fca369, Fca559, Fca742, HDZ700), bringing the total number of studied microsatellites to 19.

Genotyping of the historic and tissue samples is described in published papers (Polanc et al. 2012; Sindičić et al. 2013).

For noninvasive samples, all PCRs were done using Qiagen Multiplex PCR kit. We prepared 10 µL reactions – 5 µL of Qiagen Mastermix, 1 µL of Q solution, 2 µL of template DNA and 2 µL of primer mix and water to obtain the appropriate concentration. Microsatellites were amplified in 3 multiplexes (Polanc et al. 2012). The reactions were first denatured at 95°C for 15 minutes, then cycled for 50 cycles at 94°C for 40 seconds, 60°C,57°C or 53.8°C (depending on the multiplex) for 90 seconds and 72°C for 90 seconds. We followed this with a final extension step of 30 minutes at 60°C.

For noninvasive samples, we used a modified multiple-tube approach (Taberlet et al. 1996; Adams & Waits 2007) with up to 8 re-amplifications of each sample according to the sample's quality and matching with other samples. In the first screening process, each sample was amplified with the full genotyping PCR protocol twice and analyzed on an automatic sequencer (Applied Biosystem ABI 3130xl Genetic Analyzer). Samples that provided no specific PCR products at that stage were discarded; the other were genotyped up to eight times, with reliability of the genotype being checked with Reliotype (Miller, Joyce, & Waits, 2002) maximum-likelihood approach after each genotyping run.

Good quality tissue samples were re-amplified twice. For marginal/poor samples (e.g. decomposing tissue) we used the same approach as for noninvasive samples.

Data analysis

Genetic data were prepared in laboratory database (MisBase), which we use to keep a record of the field data. The data were exported into GIS software (QGIS) to determine spatial characteristics of each data point. All non-GIS analyses were run in R (R Development Core Team 2018).

Genetic diversity and genetic structure for all studied populations and genetic drift in the Dinaric population

Nuclear DNA diversity was measured as the number of alleles per locus (*A*), the observed heterozygosity (*Ho*) and Nei's unbiased expected heterozygosity (*He*) (Nei 1978) using the R statistical environment with package 'adegenet' (Jombart 2008). We used the same package to explore genetic structure using principal component analysis (PCA). We visually examined the eigenvalues in scree plot to determine the number of interpretable components and plotted the results to explore the patterns of genetic structure.

Effective population size

All estimates of effective population size were done in program NeEstimator (Do et al. 2014). Data was prepared in R.

We used the linkage disequilibrium method (LD) to estimate effective population size (*Ne*) of the Dinaric population and how it changed through time (Waples 2006). The method uses linkage disequilibrium between loci in a single sample of genotypes which forms between genomically unlinked loci when effective population size is not infinite. In small populations (*Ne* < approx. 500) this signal becomes strong enough to enable an estimate of the effective population size. To obtain large enough sample sizes we separated data into groups of samples by decades when the animals were sampled, with the exception of the last decade where we had a lot of samples and were able to separate the samples into two groups by time to obtain a better resolution for the period immediately before population augmentation (which is of the greatest interest to us). Extensive testing with simulations has indicated that already 25 individuals per group should provide enough data for reliable estimates when the actual effective population size is small (Waples 2006; Waples & Do 2010), and all our groups of samples exceeded this threshold. Following recommendations from Waples & Do (2010) we excluded rare alleles with frequencies below 0.02. Waples and Do (2010) discussed a reasonable conjecture that samples that include several cohorts should correspond to *Ne* in a generation, which was later supported with simulations by Robinson & Moyer (2013). As our groups of samples randomly included animals of different (unknown) age collected over a 5-10 year timespan, each sample for LDNe should include several adjacent cohorts and provide a reasonable estimate of generation *Ne*.

The second method we used was the temporal method. It builds on the actual definition of the effective population size as index describing how genetic diversity drops and inbreeding increases through time. We used Jorde-Ryman's derivation of the method (Jorde & Ryman 2007), which should provide unbiased estimates. We used the same groups of samples as for the LDNe method and calculated effective population size estimates for all of them in a pairwise fashion.

Genetic drift, inbreeding and inbreeding depression in Dinaric population

We used a travelling window analysis (Sindičić et al. 2013b) to explore erosion of genetic diversity caused by genetic drift in the Dinaric population. We obtained the dynamics of heterozygosity through time. We used 40 samples as the width of the window.

We also used PCA on Dinaric population samples separated in time to explore genetic drift. We used the same temporal groups of samples as for estimates of effective population size to explore if drift was affecting genetic structure, with the expectation being that the population would get further and further away from the source population in the PCA space as time progressed. For the same purpose we used SnapClust (Beugin et al. 2018), looking for a temporal pattern in cluster assignments of samples from different time periods.

We used Wright's hierarchical structuring of inbreeding (Wright 1931) to estimate the total inbreeding, F_{it}. As suggested by Keller & Waller (2002), in a randomly breeding population ($F_{is} = 0$) the actual inbreeding that would cause inbreeding depression (probability of alleles at a locus being identical by descent) would equal F_{st} between the studied population and metapopulation / entire species. In the case of Dinaric lynx, since the population has been reintroduced from Slovakian Carpathians, the drift component of inbreeding (F_{st}) directly indicates the inbreeding of Dinaric population relative to the source population in Slovak Carpathians. We termed this '*effective inbreeding (Fe)*' (Frankham et al. 2002), where $Fe = 1 - H_{Din}/H_{SK}$, with H_{Din} being heterozygosity in the Dinaric lynx and H_{SK} being heterozygosity in the source population in Slovakia.

A relatively recent meta-analysis of inbreeding depression in the wild indicated on average 12 diploid lethal equivalents (2B) in wildlife populations (O'Grady et al. 2006), which matches closely with F-corrected estimates published in a previous study (Crnokrak & Roff 1999). Using the formula for inbreeding depression $\delta = 1 - e^{-BF}$, where B is the number of gametic lethal equivalents and F the inbreeding coefficient, it is straightforward to estimate the expected inbreeding depression. As we estimated inbreeding relative to the source population in Slovakia, such are also estimates of inbreeding depression. Remaining relative fitness compared to Slovak lynx was calculated as $1 - \delta$.

Results and discussion

Samples

Altogether we collected and genotyped samples of 217 different lynx: 22 from Romania, 48 from Slovakia and 147 from NW Dinaric Mountains (Slovenia and Croatia). The Dinaric sample set included also historic samples analyzed in Sindičić et al. (2013) and two animals sampled close to Slovenian border in Italy (one of them translocated from Switzerland). It also included two samples from Croatia received anonymously of unverified origin, and six samples with missing data for the year of collection. The Slovak samples, besides samples from project partners, also included tissue samples provided by Ladislav Paule (Technical University in Zvolen) and noninvasive samples provided by Robin Rigg (Slovak Wildlife Society). The Romanian samples included besides the samples from project partners also samples provided by Barbara Promberger (Foundation Conservation Carpathia) and analyzed in networking with project LIFE18 NAT/RO/001082 LIFE CARPATHIA (Creation of a Wilderness Reserve in the Southern Carpathians, Romania).

Since there can be several samples collected from the same animal (particularly in noninvasive samples), only the best-amplifying sample of each animal was used, meaning that each sample in the dataset used for this analysis and reported here belongs to a different animal.

Table 1: Collected samples of individual animals, by sample type. Urine was collected in snow. Saliva direct is saliva collected directly from the mouth of animals – in telemetry/translocation captures or in sampling of kittens.

Sample Type	Number
Blood	22
Bone	9
Hair	24
Paw	48
Saliva	3
Saliva direct	8
Scat	43
Tissue	45
Urine	17
Total	219

Because of the history of the population in NW Dinaric Mountains, we can expect small effective population size and strong genetic drift. For this reason, we must take into consideration the time component in analyses of genetic data from that area. Temporal distribution of samples from that area is shown in Figure 1.

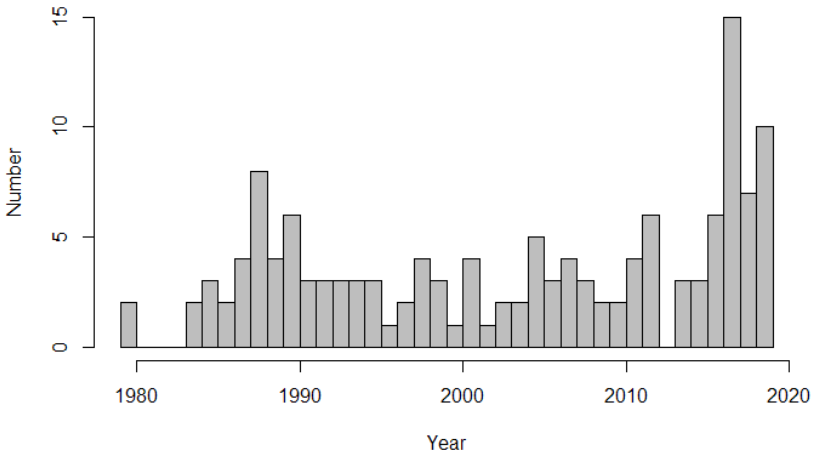

Figure 1: Temporal distribution of successfully genotyped lynx samples from Dinaric Mountains. Each sample is from a different animal.

Genetic structure between study areas and genetic diversity

Study areas in Romania and Slovakia are spatially relatively far from one another, so it's reasonable to expect a certain level of genetic structuring. But nevertheless the lynx in Romania and Slovakia should still be a part of the same Carpathian population (Ratkiewicz et al 2012; Forster et al 2018) Similarly, since the population in Dinaric Mountains went through a population bottleneck, both bottleneck sampling in the 1973 reintroduction (with only 6 related animals not all alleles were transferred to Dinarics, and allelic frequencies were changed) and subsequent genetic drift should create detectable genetic structuring.

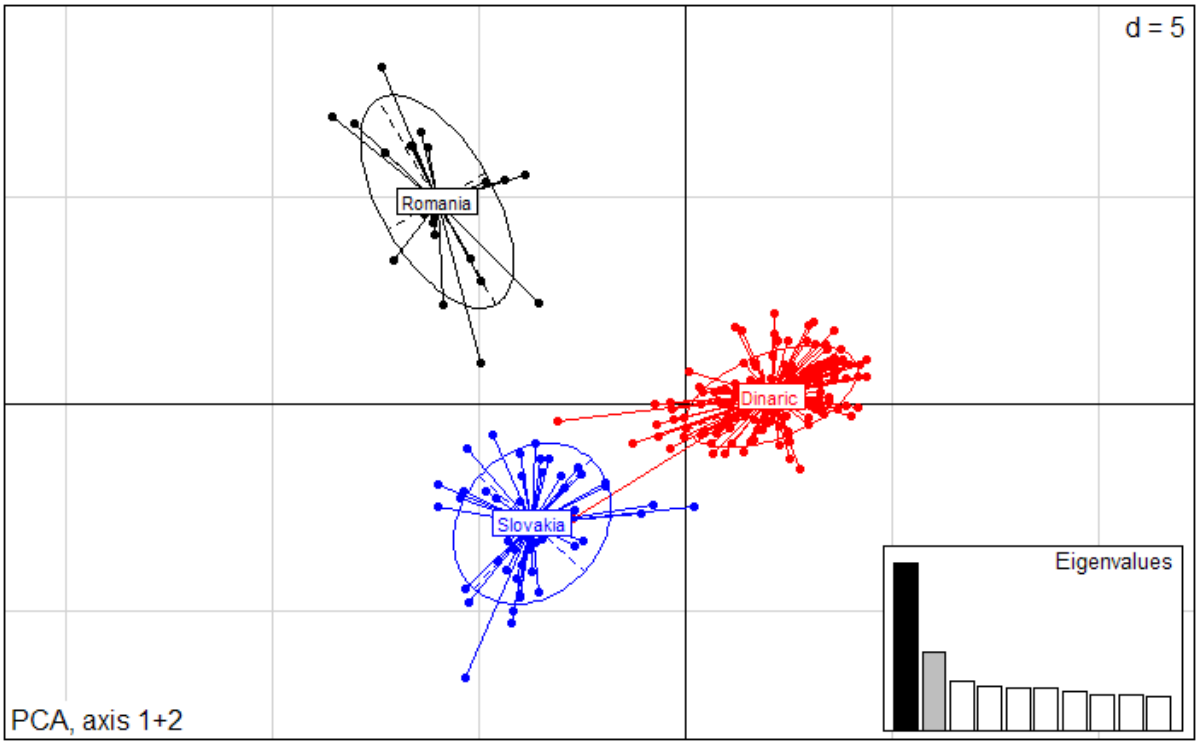

Figure 2: Principal component analysis of microsatellite data. Axes 1 and 2 (with high eigenvalues) show clear structuring both within the Carpathians as well as the structure caused by reintroduction sampling / genetic drift in Dinaric Mountains.

As we can see in Figure 2, a simple PCA of the genetic data clearly separates all three populations on two PCA axes. There are two animals (Figure 3) that were sampled as "Dinaric" but cluster with or close to the source population in Slovakia. One of these lynx (R18) was killed (legal hunting) in 1990 and may have been an old lynx born in some of the first generations after the reintroduction, and the other (M21EX) was an animal translocated from Switzerland to Italy (lynx were introduced to Switzerland also from Slovakia). There is another animal (M1YEA) that clusters half-way between the Slovak and Dinaric samples. This sample (tissue) was obtained anonymously in Croatia in 2016. Since we don't have any other data about the sample and we can't verify its authenticity, it was removed from downstream analyses.

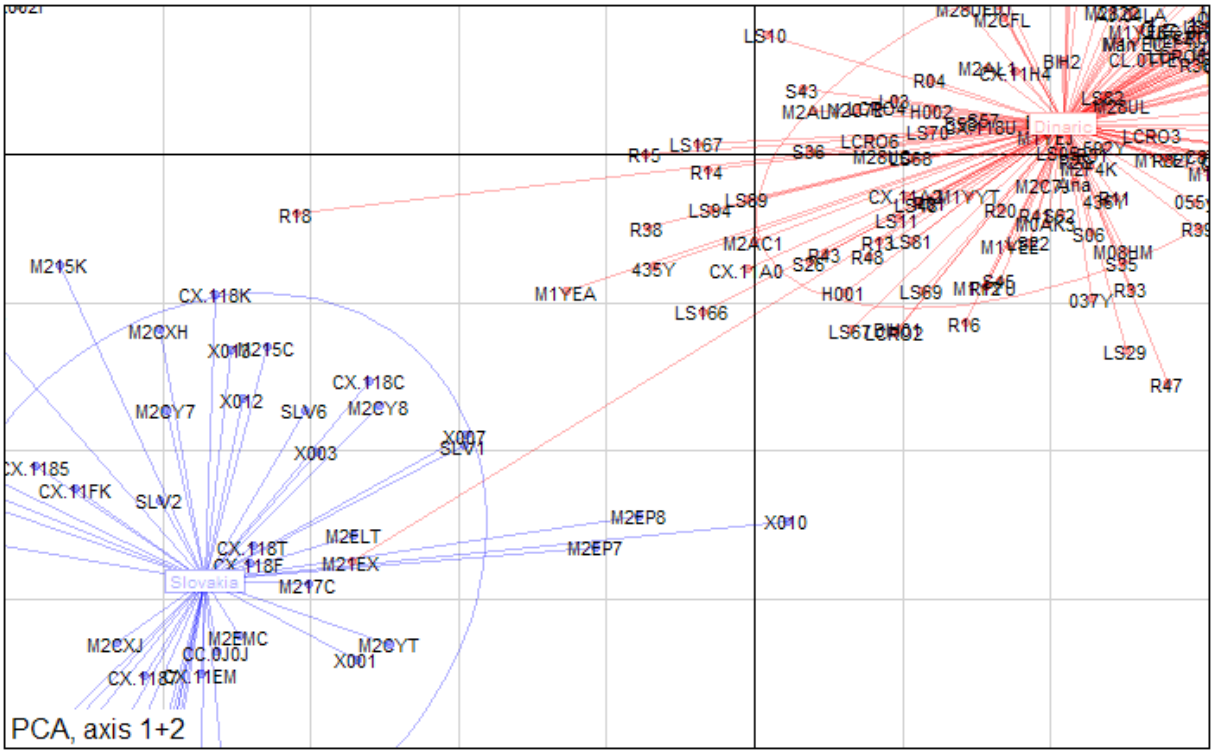

Figure 3: Principal component analysis of microsatellite data, closeup. Two "Dinaric" animals that cluster with Slovak samples are a lynx translocated to Italy from Switzerland and an animal from Slovenia from 1990 (possibly an older animal).

Heterozygosity in Slovakia and Romania are considerably higher than in the Dinaric population (Table 2), although for Romania we still have a relatively small sample size. This is particularly reflected in allelic diversity, which is in Romanian samples probably lower because of the small number of samples. We can also see that Dinaric population is losing its genetic diversity through time.

Table 2: Genetic diversity indices for the three study areas and changes in diversity indices in Dinaric Mountains through time. N = Number of animals, He = expected heterozygosity, Ho = observed heterozygosity, A = allelic Diversity, SE = Standard error, N HW = number of loci out of Hardy-Weinberg equilibrium at p = 0.05. N markers = 19. 10 Dinaric samples with insufficient data or other problems were removed from these calculations.

Population	***N***	***He***	***SE He***	***Ho***	***SE Ho***	***A***	***SE A***	***N HW***
Slovakia	48	0.583	0.031	0.585	0.034	4.21	0.181	2
Romania	22	0.545	0.039	0.512	0.040	3.58	0.257	3
Dinaric	139	0.457	0.036	0.429	0.036	3.21	0.282	6
Dinaric Mountains, by time periods.								
Time period	***N***	***He***	***SE He***	***Ho***	***SE Ho***	***A***	***SE A***	***N HW***
1979-1990	31	0.481	0.036	0.439	0.036	2.79	0.164	1
1991-2000	26	0.471	0.036	0.477	0.042	2.95	0.209	1
2001-2010	28	0.432	0.040	0.431	0.046	2.74	0.200	1
2011-2016	22	0.397	0.038	0.390	0.039	2.58	0.159	0
2017-2019	32	0.399	0.039	0.407	0.042	2.63	0.175	1

Effective population size of the Dinaric lynx population

Different methods of determining effective population size provide estimates for different time periods and differ in their sensitivity to violation of assumptions. We organized genotypes by the year of sample collection into four samples (periods): 1979-1990, 1991-2000, 2001-2010 and 2011-2019 for the temporal method. Effective population size is estimated from changes in allelic frequencies between pairs of samples separated in time.

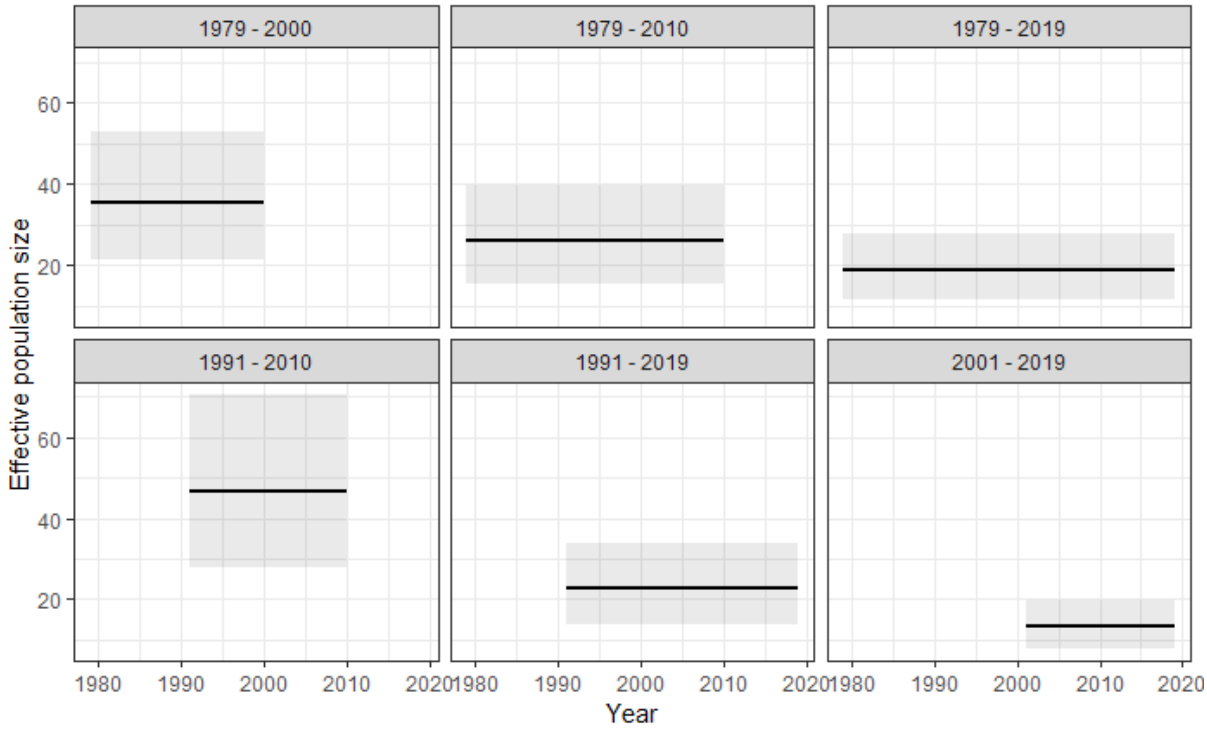

Figure 4: Effective population size (Ne) determined with the temporal method for different time periods. The timespan indicates the year of the first sample and the last sample, the band indicates the parametric confidence interval. The estimated Ne should be the harmonic mean effective population size of the period in question.

Figure 4 and Table 3 show the results of the temporal method for estimating *Ne*. The best estimate of *Ne* with the temporal method is from the first until the last sample – for the 1979 to 2019 period. As the time period is the longest, the changes in frequencies of alleles are the highest, meaning that the estimate is based on the strongest signal data. Being the harmonic mean, it is weighted towards the lowest effective population size in the period. However, all estimates seem useful - since the effective population size is small, genetic drift is strong, and even with short time between samples the changes in allelic frequencies were high enough to provide believable estimates of *Ne*.

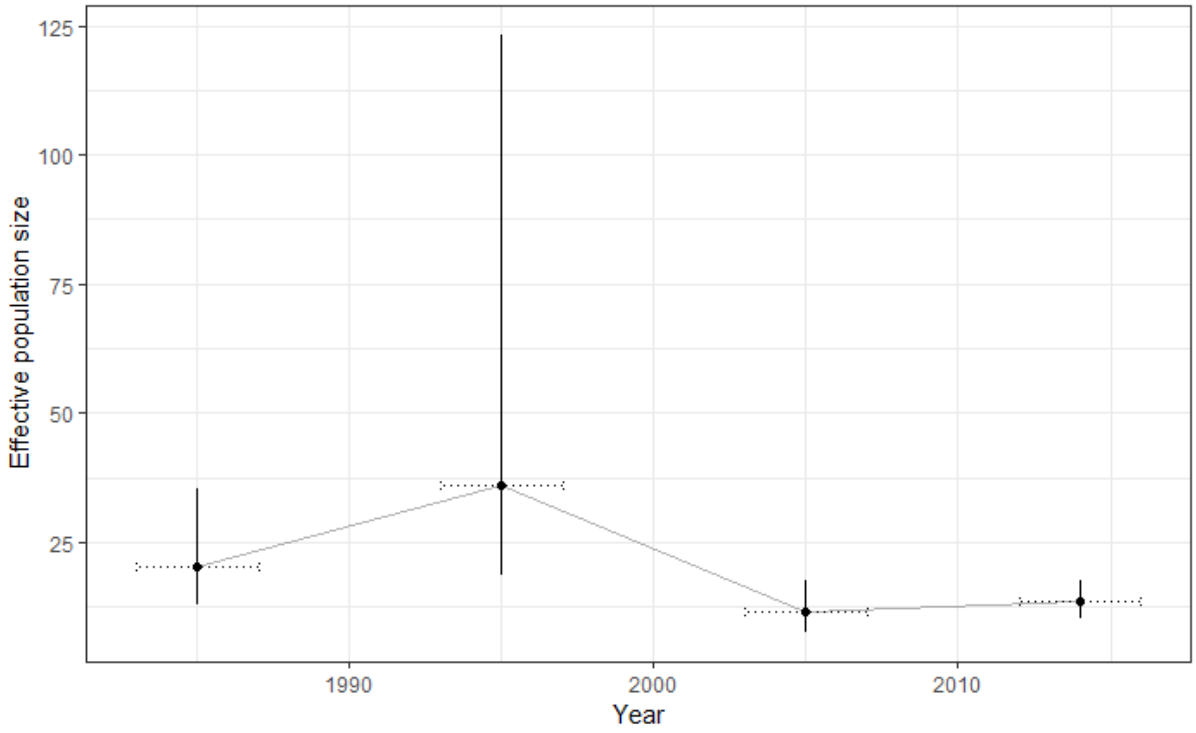

Figure 5: Effective population size estimates with the linkage disequilibrium (LDNe) method. Vertical lines indicate 95% confidence intervals, horizontal dotted lines indicate approximate timespan the estimates apply to.

Figure 5 and Table 3 show the effective population size estimated with the linkage disequilibrium (LDNe) method. This method is conceptually quite different than the temporal method since it uses a different phenomenon as a signal (linkage disequilibrium between loci in the same set of samples vs. changes in allelic frequencies of the temporal method) and uses the data in a very different manner. It is encouraging that results obtained by both methods are comparable, which increases their credibility.

Table 3: Estimates of effective population size obtained for different time periods by different methods. The table is sorted by the time period. Ne = effective population size, CI = 95% confidence interval. The 1979 – 2019 temporal method estimate (underlined) indicates the harmonic mean Ne for the entire period from soon after the reintroduction until 2019. The 2011-2019 LDNe estimate (bold) indicates the "final" estimate of effective population size before the start of population augmentation in 2019.

Period (First - last samp.)	Method	Ne	Parametric CI	Jackknife CI
1979 - 1990	*LDNe*	*20.3*	*12.9 - 35.5*	*11.4 - 43.6*
1979 - 2000	Temporal	35.3	21.3 - 52.8	19.2 - 223.1
1979 - 2010	Temporal	26.3	15.5 - 39.9	16.2 - 69.1
1979 - 2019	Temporal	18.7	11.4 - 27.8	10.5 - 88.1
1991 - 2000	*LDNe*	*35.9*	*18.7 - 123.2*	*17.3 - 182*
1991 - 2010	Temporal	46.7	27.7 - 70.5	20.0 - Inf
1991 - 2019	Temporal	22.6	13.7 - 33.7	12.8 - 96
2000 - 2010	*LDNe*	*11.4*	*7.6 - 17.5*	*7.5 - 17.8*
2001 - 2019	Temporal	13.3	8 - 19.9	6.9 - 219.1
2011 - 2019	***LDNe***	***13.4***	***10.2 - 17.5***	***8.1 - 21.4***

The time period that an estimate applies to is not always straightforward to determine. While the temporal estimates provide a harmonic mean of *Ne* between two sampling periods, the samples were not collected "momentarily", but rather include the animals collected within approximately a decade (~2-2.5 lynx generations). LDNe estimates, on the other hand, also include the samples from approximately 1 decade and should indicate *Ne* of the parental generation (shift of 4-5 years). With several generations included in the estimate, it should approach the actual population *Ne*.

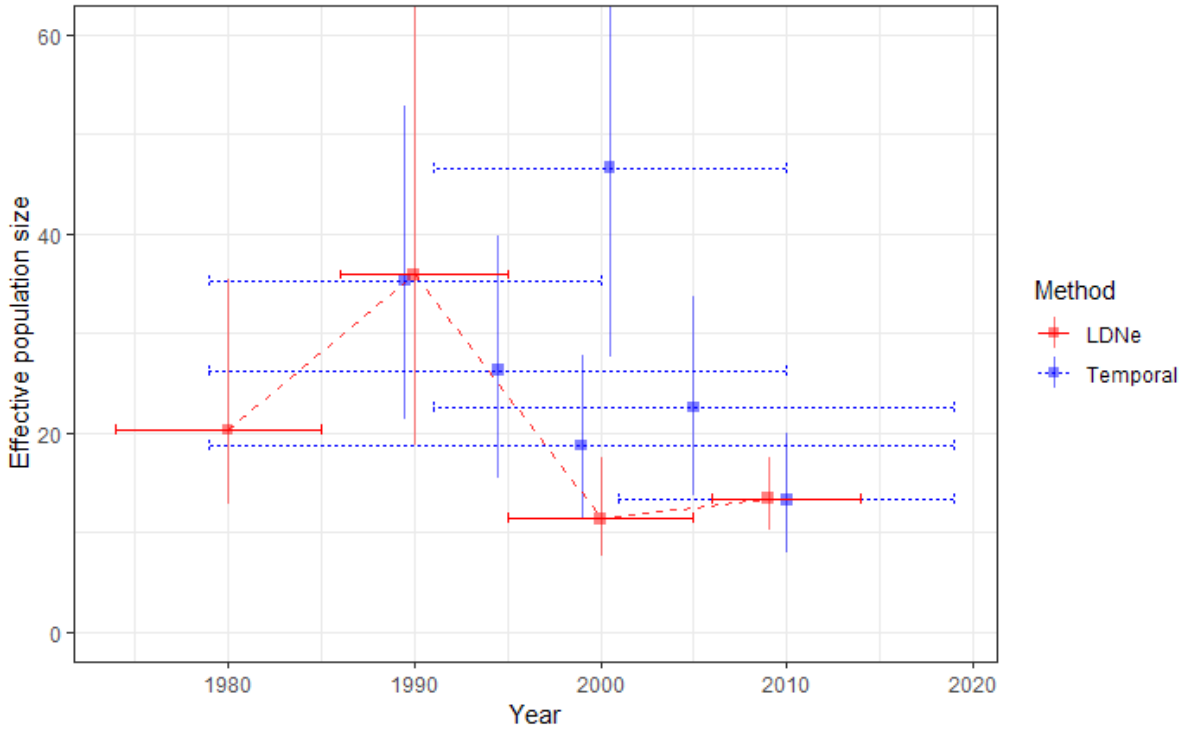

Figure 7: Effective population size estimated for both methods for different time periods. Confidence intervals are shown as vertical bars. The time period an estimate should apply to is indicated by horizontal bars.

It seems that while the effective population size decreased over the entire period, it was higher when the population went through the period of rapid expansion after the 1973 reintroduction. In a growing population less genetic diversity is lost than in a stable or declining population, hence the higher effective population size. The effective population size later dropped, and now seems to be at approximately the same level (13.4, 10.2 - 17.5 95% CI) for the last two decades. This is far from the rule-of-the-thumb threshold of the minimum for population viability (*Ne* > 50), and if it stays the same, we can expect 3.7% (2.9 – 4.9% CI) drop in genetic diversity and increase in inbreeding per generation.

Genetic erosion of Dinaric lynx population – decrease of genetic diversity, increase of inbreeding and expected inbreeding depression

In populations with low effective population size genetic drift becomes the main evolutionary force shaping the genetic outlook for the population. Such populations rapidly lose genetic diversity and become inbred, and Dinaric lynx are no exception.

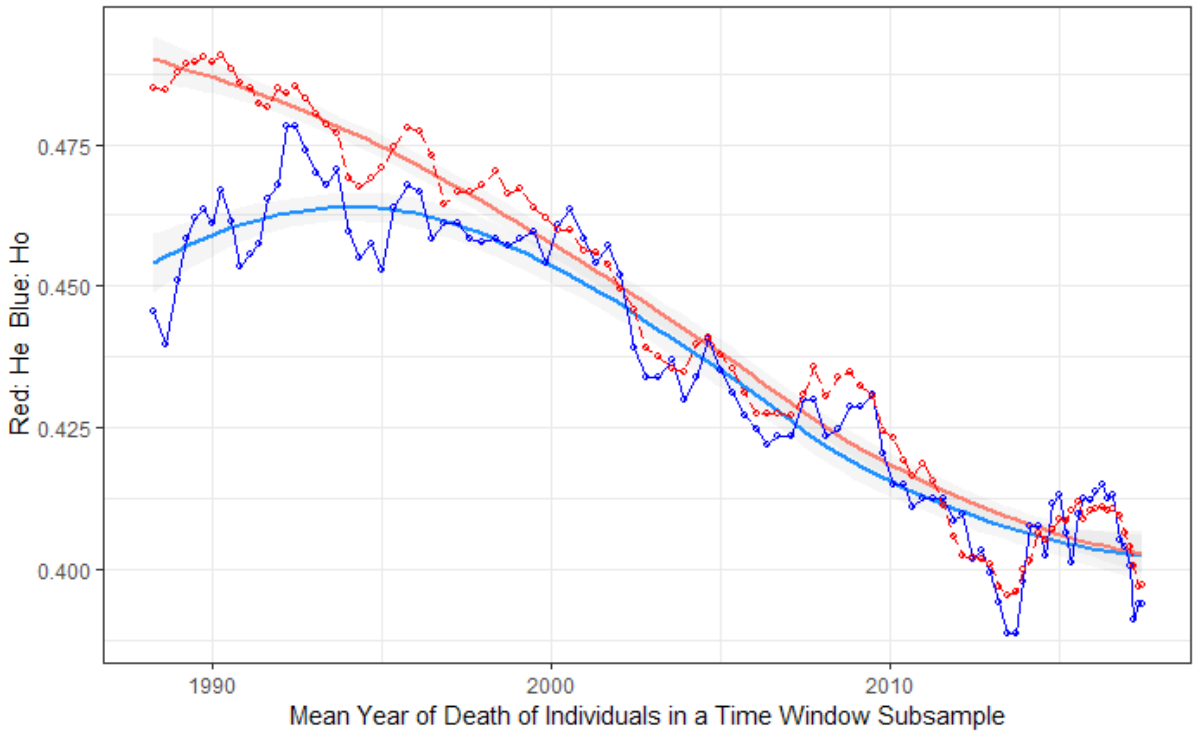

Figure 7: Decline in heterozygosity in Dinaric lynx – travelling window analysis, each point is an estimate of 40 samples neighboring in time.

Figure 7 shows decline in heterozygosity in Dinaric lynx. As expected with the small effective population size, the decline was rapid. In the beginning there is a difference between observed and expected heterozygosity. In the first generations after the 1973 reintroduction, the population was small and there was no other option than breeding with close relatives, causing Fis > 0 (assortative mating) in the population. Later, as the population grew, the mating became random, but inbreeding increased as an effect of genetic drift.

We can also clearly see genetic drift in genetic structure analysis (Figure 8) where through years the Dinaric population genetically drifts further and further away from the source population.

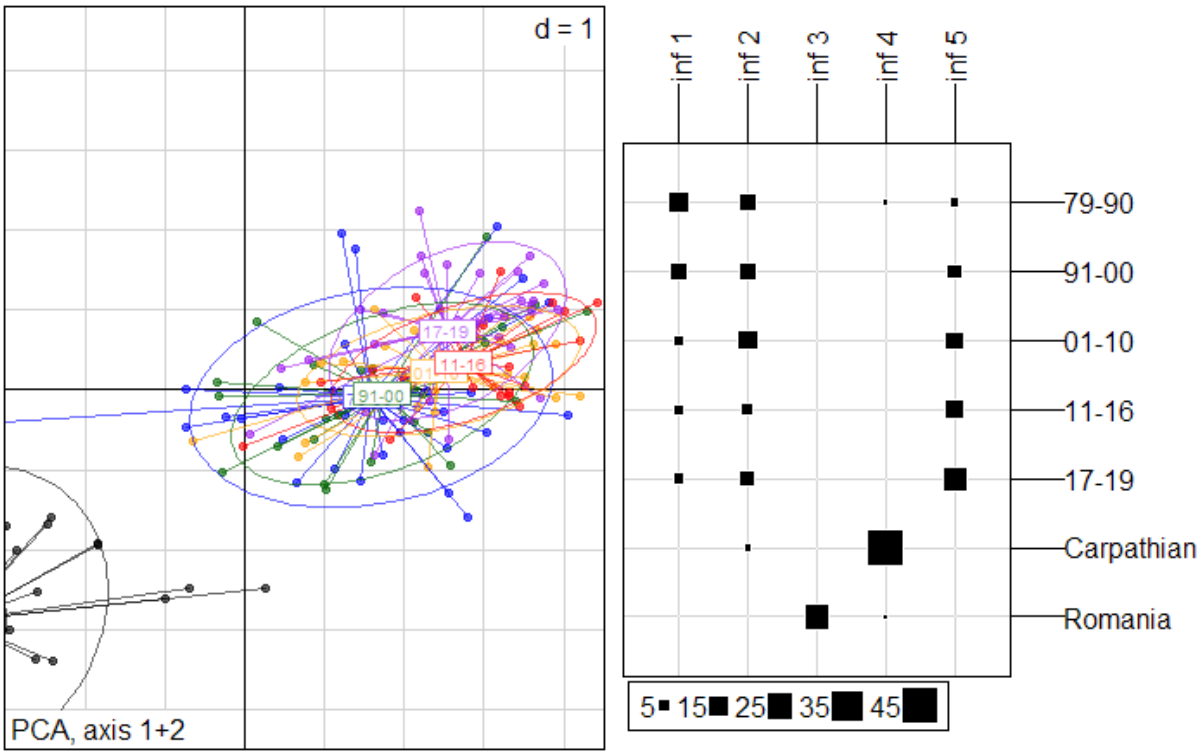

Figure 8: Genetic drift in the Dinaric lynx population. Left: PCA analysis, Dinaric population colored by the period in which the samples were collected. We can see that population genetically 'drifts' away from the source population in Slovak Carpathians (bottom left, black). Right: SnapClust genetic clustering (Beugin et al. 2018). *Inferred genetic clusters are vertical (on top) and sampled populations horizontal (right). Dinaric population samples were separated by decades. We can see the Dinaric population 'drift' through time from inferred clusters 1 and 2 in the first decades towards the inferred cluster 5 in the final decades.*

Since the population was reintroduced, and F_{is} seems to be close to zero, we can estimate the total inbreeding in the population as the genetic drift from the source population as 'effective inbreeding', Fe. Results are shown in Table 4 and Figure 8.

Table 4: Heterozygosity, effective inbreeding (Fe) and expected inbreeding depression (δ) in Dinaric lynx.

Time period	N	*He*	*Fe*	*δ*
Slovakia (source)	48	0.583	0	0
NW Dinaric Mts.				
1979-1990	31	0.481	0.176	0.653
1991-2000	26	0.471	0.192	0.684
2001-2010	28	0.432	0.260	0.789
2011-2016	22	0.397	0.319	0.853
2017-2019	32	0.399	0.316	0.850

Estimates of inbreeding depression are provided in Table 4, and dynamics in Figure 9.

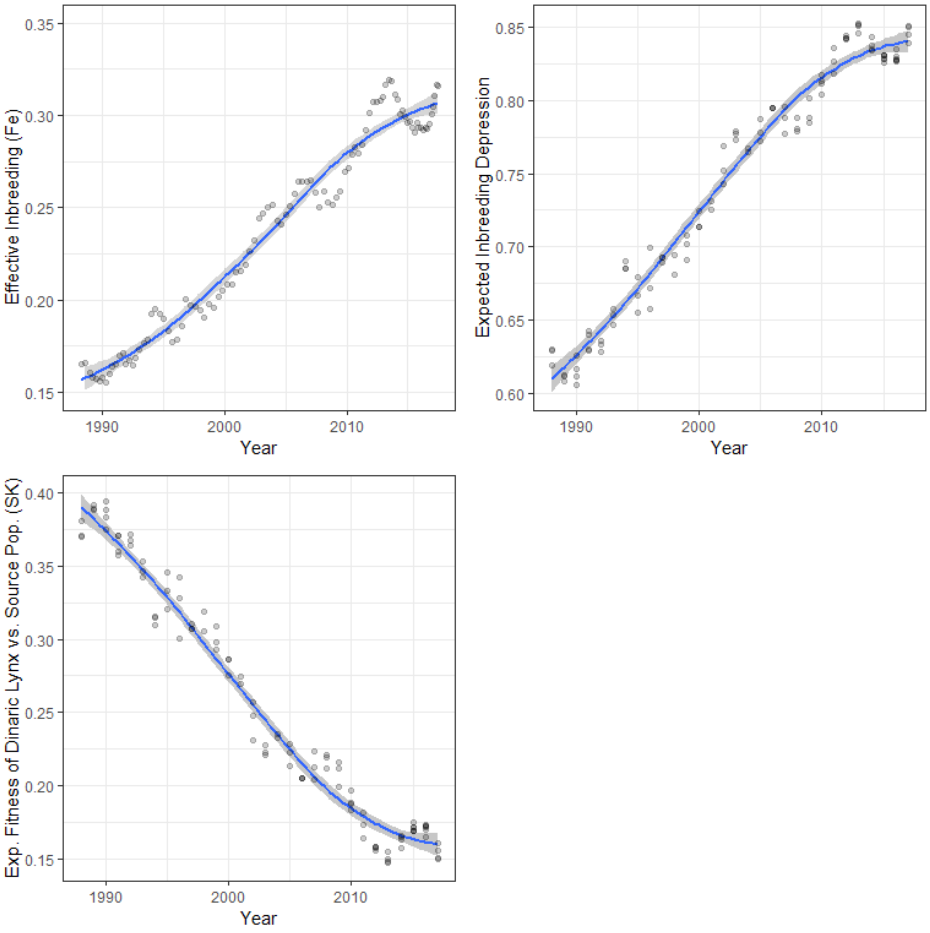

Figure 9: Effective inbreeding (upper left), expected inbreeding depression (upper right) and expected remaining fitness of the Dinaric lynx population (lower left) relative to the source population in Slovak Carpathians. We used 12 diploid lethal equivalents (O'Grady et al. 2006) *to estimate inbreeding depression.*

Even in the 1980s, relatively soon after the reintroduction, inbreeding is already relatively high (*Fe* = 0.176), as is the expected inbreeding depression (δ = 0.653). In the favorable environment, the population was able to compensate for these negative effects and was doing well at the time (Kos et al. 2004). However, in the 2001 – 2010 period, inbreeding in the population increased over that of a brother – sister mating (*F* = 0.260), with expected inbreeding depression becoming very high (δ = 0.789). We can expect that in this period, both viability and fecundity of Dinaric lynx were already severely impacted by genetic erosion of the population. This process continued, and for the last measurement period 2017 – 2019, we see inbreeding reach *F* = 0.316 with corresponding expected inbreeding depression δ = 0.850. This means that the expected remaining fitness of the Dinaric lynx population compared to the source population in Slovak Carpathians is in this last period 0.15.

There are a couple of caveats in this analysis. First, the actual effects of inbreeding on fitness vary considerably between species and populations. That said, they are never negligible, particularly a wild population, and have been pretty much shown in all cases that had sufficient statistical power (Keller &

Waller 2002; Reed & Frankham 2003; Hedrick & Garcia-Dorado 2016). Second, in the years since the reintroduction, especially since the population initially grew in a favorable environment, there could have been some purging of lethal recessive alleles. However, the load of non-lethal deleterious recessive alleles still remains in the population with such small effective population size as it is impossible to purge any alleles that have selection coefficient below 1/2Ne. Added to that is also the 'fixation load' of semi-recessive deleterious alleles that get fixed in such small population because of genetic drift. All in all, considering the high inbreeding in the Dinaric lynx population, we can expect the actual inbreeding depression to be considerable and quite probably endangering population's survival.

Conclusions

The genetic picture of the Dinaric lynx population in the final years before population reinforcement appears grim. Although the population did well soon after the 1973 reintroduction, which is indicated also in its relatively high effective population size estimates from that period indicative of population expansion, it lost considerable proportion of genetic diversity already through the reintroduction bottleneck. High genetic drift caused by small effective population size and limited number of unrelated mates immediately after the reintroduction caused rapid inbreeding, followed in-step by the related drop in heterozygosity. While it seems that the population was still doing well in the 1980s when inbreeding coefficient is estimated at *Fe* = 0.176, by 1990s this parameter reached *Fe* = 0.192. At this level of inbreeding we can already expect 68% drop in fitness (δ = 0.684) at the population level. Although not yet obvious in the field, we can speculate that inbreeding depression may have already started to have a pronounced effect on demography. By the 2000s, when field reports started indicating decreasing lynx population, inbreeding already reached *Fe* = 0.26, meaning that on average the Dinaric lynx were more inbred than offspring produced by mating between brothers and sisters. The expected drop in fitness would be 80%. In the last three years before the 2019 population reinforcement, inbreeding reached *F* = 0.316, with corresponding expected drop in fitness of 85%.

Despite these data and clearly high levels of inbreeding, the actual inbreeding depression is hard to determine. The "smoking gun" is in the case of inbreeding often a very hard thing to find, particularly if the population is small and difficult to study. However, there were three lynx found dead in Slovenia over the last decade because of generalized sepsis, a very rare condition in carnivores that may indicate some problems with immunity. While there were almost certainly few lethal recessive alleles in the population (alleles that cause death of the individual when homozygous), these may have been purged since they are very strongly selected against. This may actually have happened, as the drop in genetic diversity seems to have slowed down towards the end of the studied period (Figure 7), and the estimate of the effective population size is somewhat higher (Figures 5 and 6, although there is no statistical significance in this difference). However, the load of recessive deleterious alleles remains, as well as the "fixation load" of slightly deleterious semi-recessive or codominant alleles that got fixed in the population because of genetic drift. With small effective population size, the selection would need to be extremely strong for any allele to be selected for or against. For example, with *Ne* = 13, the selection coefficient should be $s > 0.038$ for an allele to be visible to selection, which is very unlikely for any alleles save for the lethal recessives. In the optimistic scenario of 5 lethal equivalents caused by lethal recessive alleles being purged by selection (O'Grady et al. 2006), the expected inbreeding depression for the 2017 – 2019 period would still be δ = 0.669, or 66.9% drop in fitness relative to the source population.

All in all, the population has not been doing well from the genetic perspective, and as the field data indicate, was going into the "extinction vortex" (Frankham et al. 2002). It's difficult to predict when the population would go extinct without intervention, but there is little doubt that extinction would be a matter of "when" rather than "if". While the genetic restoration of the population carries its own caveats (Tallmon et al. 2004; Hedrick 2005; Hedrick & Garcia-Dorado 2016), the chances of survival of the Dinaric lynx should improve with each new translocated animal. But even while LIFE Lynx should save the Dinaric lynx population from immediate extinction, it's not the "final solution". The population will need genetic management in the long run to maintain its viability. The experiences obtained through the project will be invaluable in planning such genetic management, and a firm understanding of the "baseline" status immediately prior to population augmentation that this study provides is the critical first step.

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