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

Development of the population and impact of the reinforcement program with experiences gained and recommendations for future lynx reinforcement projects

Action D.2

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Introduction

The current Dinaric lynx population originates from the reintroduction in 1973. Six animals were brought from Slovak Carpathians and released in south-eastern Slovenia close to town Kočevje. In the beginning, the reintroduction was very successful, and the population rapidly grew and expanded (Čop & Frković 1998; Kos et al. 2004). But only six animals founded this population (some of them even related), and they remained isolated with no gene flow from other populations. The lynx in NW Dinaric Mountains soon had no other option than to mate with relatives, and inbreeding started accumulating. In comparison with other reintroduced lynx populations in Europe, this one had the lowest number of founders and was the most inbred (Müller et al., 2022).

The population decline started to be observed in the 2000s (Kaczensky et al. 2012) and by the 2010s signs of lynx presence in the field became increasingly rare. The pre-reinforcement genetic study (Skrbinšek et al., 2019) showed that 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 inbreeding coefficient considerably exceeded the 0.25 limit proposed by the Bonn Lynx Expert Group for the conservation of the Eurasian lynx, when immediate action should be taken (Bonn Lynx Expert Group, 2021).

The analysis of all the data collected immediately prior to the reinforcement gave us an overview of the status of the Dinaric lynx population. This baseline allows us to evaluate the effects of the lynx reinforcement and assess the final population status.

With the beginning of the first translocations in the Dinaric area as well as the establishment of the lynx population stepping stone in the SE Alps, considerable effort was devoted to monitoring of the population. In this document we combined and analyzed the data collected before the reinforcement (action A3), during the reinforcement (action C5) and after the reinforcement was completed (action D2). For population monitoring we used the data from genetic sampling and camera-trapping, and the information collected through monitoring of individual animals with GPS telemetry. At the same time monitoring was also conducted in the donor areas in Slovakia and Romania, where lynx were being captured for translocation (action D1).

The first effects of the reinforcement are already detectable. The distribution area of the lynx has increased, new areas are being recolonized, and we're seeing an increase in population density. There are more observations of signs of lynx presence. We are also documenting a decrease of inbreeding, which is now below the critical level, and the corresponding fitness is expected to be more than double its pre-reinforcement value. However, these effects should become more prominent over the next few years as the translocated animals spread their genes in the target population. Also, the stepping stone population in the SE Alps is still limited to an isolated area, and the effects of connecting the Alpine and Dinaric areas are yet to be seen.

Methods and results

Genetic data

Samples collection

In the period between the first research of Dinaric lynx population genetic diversity (Sindičić et al. 2013) and the start of the LIFE Lynx project, genetic samples were collected opportunistically. The collection of samples intensified with the start of the project in 2017 and was most intensive within the C5 action (from 2019-2023) but also continued until the end of the project. The number of collected samples per year is presented in Figure 1.
Historical genetic data and samples collected up to 2019 were already used to assess the baseline (pre-reinforcement) genetic status of SE Alpine and Dinaric Lynx population (Skrbinšek et al., 2019). In this report we focus on the genetic samples collected in the SE Alpine and Dinaric region, in Slovenia, Croatia and Italy (Figure 2) and analyzed within the LIFE Lynx project. Altogether 760 genetic samples were analyzed.

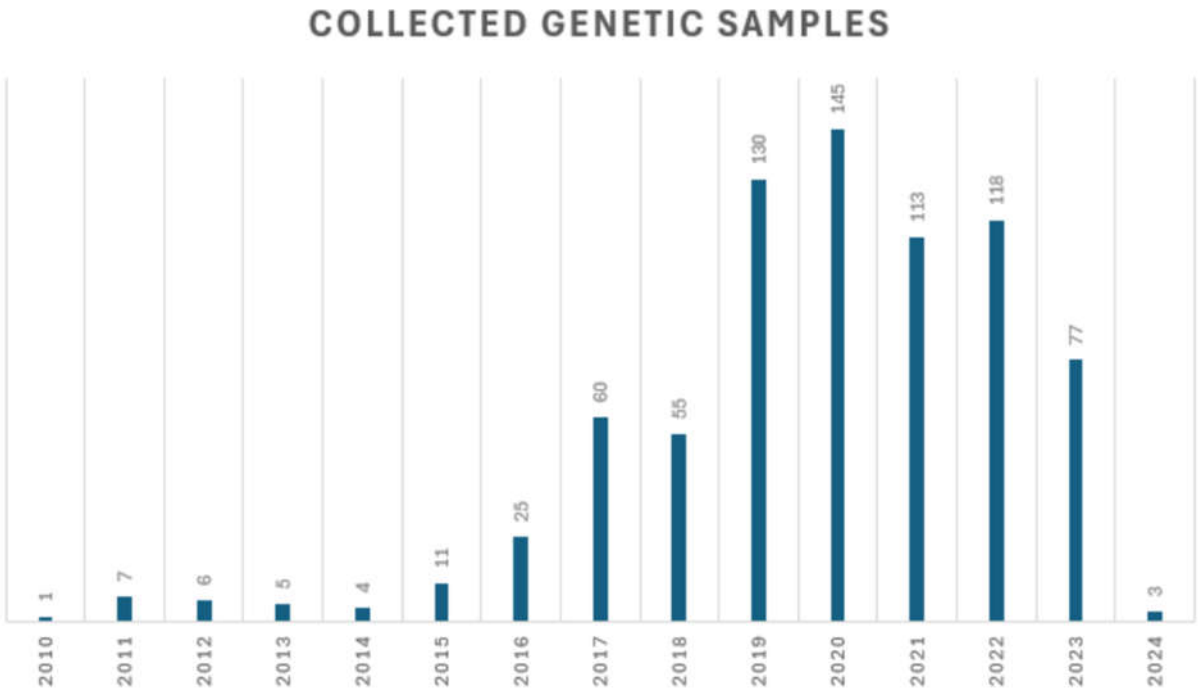

Figure 1: Number of genetic samples collected per year. Samples were analyzed within actions A3 (2010-2019), C5 (2019-2023) and D2 (2023-2024).

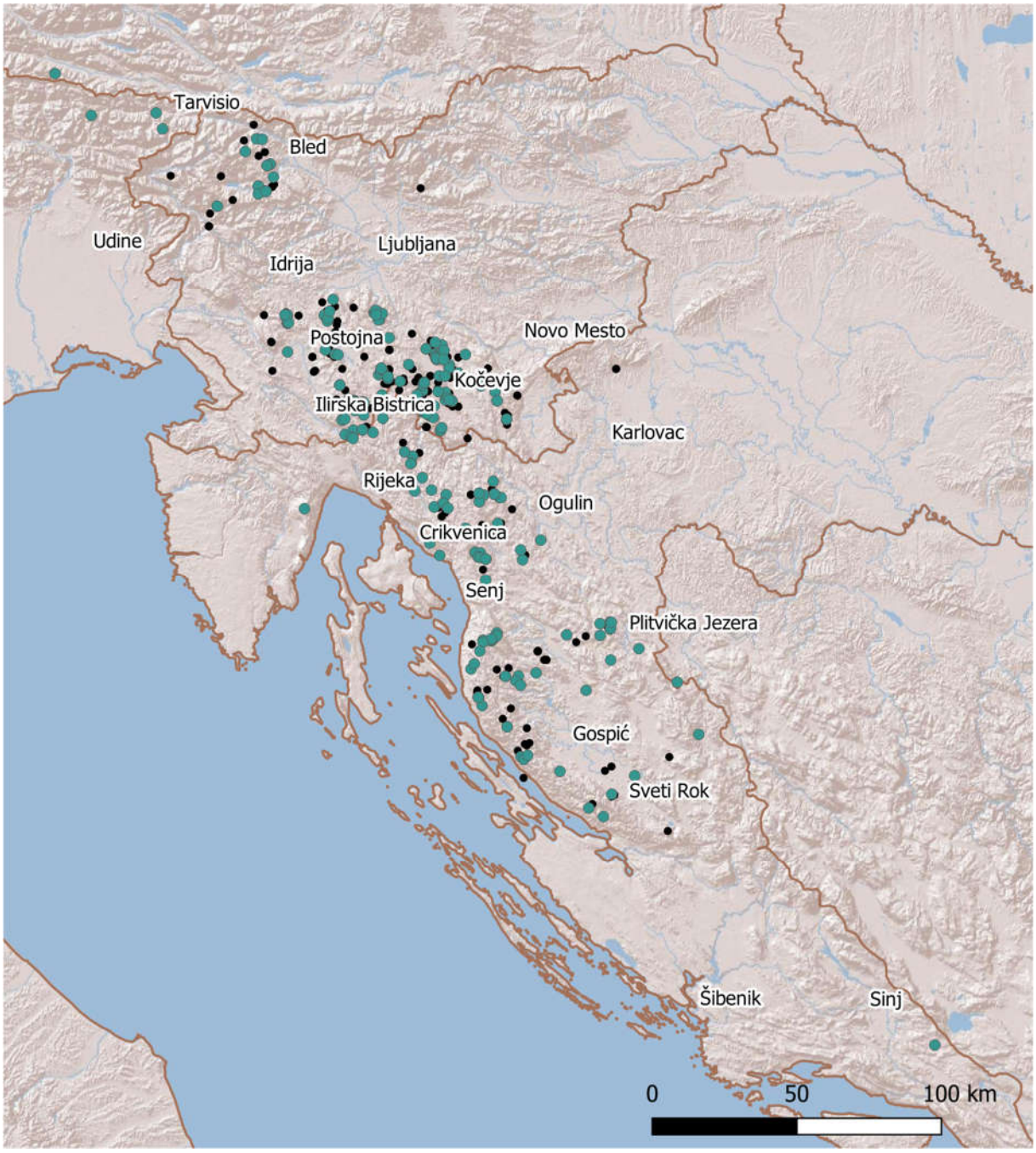

Figure 2: A map of collected genetic samples. Successfully genotyped samples are marked with green, the discarded samples with black.

Non-invasive genetic samples were collected mostly in winter during snow tracking, by visiting known marking sites or sampling lynx kills which belonged to unknown lynx. The effort needed to find lynx tracks in the snow is high, and significant manpower is needed to collect the samples. In C5 action in the second monitoring season we started collecting information on snow tracking effort in Slovenia throughout the entire monitoring season with suitable snow conditions. Also, similar reports were collected in Croatia in 2023, but limited to the Velebit

Mountains where snow cover appeared. Besides being able to track lynx, snow cover is also needed for collecting urine samples in snow. The novel environmental DNA (eDNA) method of collecting snow tracks also depends on snow conditions, but samples can be collected as soon as snow tracks are found and offer an additional source of genetic material (De Barba et al., 2024). In general, the number of collected genetic samples to a large degree depends on snow conditions, and may vary from season to season. In Table 1 we present the number of collected samples per monitoring season (1st of May until 31st of April in next year). The results are summed up for 2019-2023 from the C5 reports (Krofel et al., 2021, Fležar et al., 2022, Fležar et al., 2023 and Fležar et al., 2024).

Figure 3: Photos of genetic sampling.

Table 1: Number of collected genetic samples, with the number of successfully genotyped samples in parenthesis. The second row shows the number of identified lynx individuals per monitoring season (1st of May until 31st of April in next year). The results are summed up for 2019-2023 from the C5 reports, the time of the reinforcement of the population.

	Monitoring season			
	2019-2020	2020-2021	2021-2022	2022-2023
Total genetic samples collected (genotyped)	130 (56)	149 (71)	104 (56)	95 (36)
Nb. of identified individuals	26	31	29	21

Genotyping

Tissue samples from dead lynx were stored in 95% non-denatured ethanol and stored at -20°C. Blood samples were taken from lynx captured for telemetry. Blood was preserved on blood stain cards (Qiagen). These high-quality DNA samples were processed in a dedicated laboratory at UL.

Noninvasive genetic samples: 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. Scats were collected in ethanol. Sampling material was prepared and distributed at the beginning of the project, instructions for collecting were presented in dedicated Guidelines produced in A3 (Skrbinšek 2017). Additionally, we also collected and analyzed environmental DNA samples (eDNA). This is a novel method for genotyping individuals from the eDNA collected in snow tracks. DNA in noninvasive, historic genetic and eDNA samples is of very low quality and quantity, and contamination (especially with PCR products) is a serious issue. We used a dedicated laboratory for samples storage, DNA extraction and PCR setup.

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), For noninvasive samples analyzed within the LIFE Lynx project, we used MagMAX DNA Multi-sample Kit (Thermo Fisher Scientific). The extraction protocol is implemented on a liquid handling robot (Hamilton Starlet), samples IDs are read and handled through barcodes. The liquid handling robot is located in the "noninvasive genetics laboratory" and used exclusively for noninvasive samples.
We used ten microsatellite markers for individual ID run in a single multiplex: Fca132, Fca201, Fca247, Fca293, Fca391, Fca424, Fca567, Fca650, Fca723, Fca82. The best (reference) sample of each detected animal was amplified using 9 additional markers (F115, F53, Fca001, Fca132, Fca161, Fca369, Fca559, Fca742, HDZ700 (Menotti-Raymond et al. 1999; Menotti-Raymond et al. 2005; Williamson et al. 2002), bringing the total number of studied microsatellites to 19. SRY locus was used to determine sex of the animal. Microsatellites were amplified in 3 multiplexes, using Platinum multiplex PCR Master Mix (ABI). Protocols from Polanc et al. (2012) were adapted according to the Platinum kit user guide. The SRY sex marker amplifies also in non-felid species, and it is used for sex identification also for other carnivores, so prey DNA (like fox) in a scat could cause problems. Also, slight contamination from different animals in a sample (urine, hair, saliva from an object), can make the sex determination difficult. That is why in some cases we additionally analysed the sex of the animal with amelogenin genetic marker (Pilgrim et al. 2005). Good quality tissue and blood samples were re-amplified twice. For non-invasive 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 10-marker panel (multiB panel) protocol twice and analyzed on an automatic sequencer (Applied Biosystem ABI 3500 Genetic Analyzer). Results were interpreted using GeneMapper v.6.0. software (Applied Biosystems, USA). Samples that

provided no specific PCR products at that stage were discarded. Consensus genotypes were determined using an Access database application programmed by T. Skrbinšek (MisBase, unpublished).

Table 2: Number of collected genetic samples collected per sample type. Samples were analyzed within actions A3, C5 and D2. Only samples where individual lynx were identified are reported here.

Sample type	Count of SType	Number of genotyped lynx samples	Genotyping success
Hair	301	119	40%
Scat	203	94	46%
Urine	99	44	44%
Saliva from prey	61	14	23%
Blood noninvasive	5	3	60%
Snowtracks	9	4	44%
Saliva direct	29	25	86%
Tissue	21	21	100%
Blood	32	32	100%
Grand Total	**760**	**355**	**47%**

In Table 2 are presented numbers of collected samples per sample type and genotyping success. Only samples where individual lynx were identified are reported here as successfully genotyped samples. Total genotyping success is 47%, which is comparable with other lynx studies (Krojerova-Prokešova et al., 2019). Out of all samples, 25 samples were identified as wild cats, a non-target species that can be identified with this set of genetic markers. Including the wild cats, the genotyping success of this study increases to 51%. For identifying the species ID of the collected samples more universal genetic markers would need to be applied (eg. mtDNA). The SRY sex marker amplifies also in non-felid species, and it is used for sex identification also for other carnivores. In the data set there are samples where only SRY marker amplified well, so it cannot be excluded that these are samples of males of non-target species.

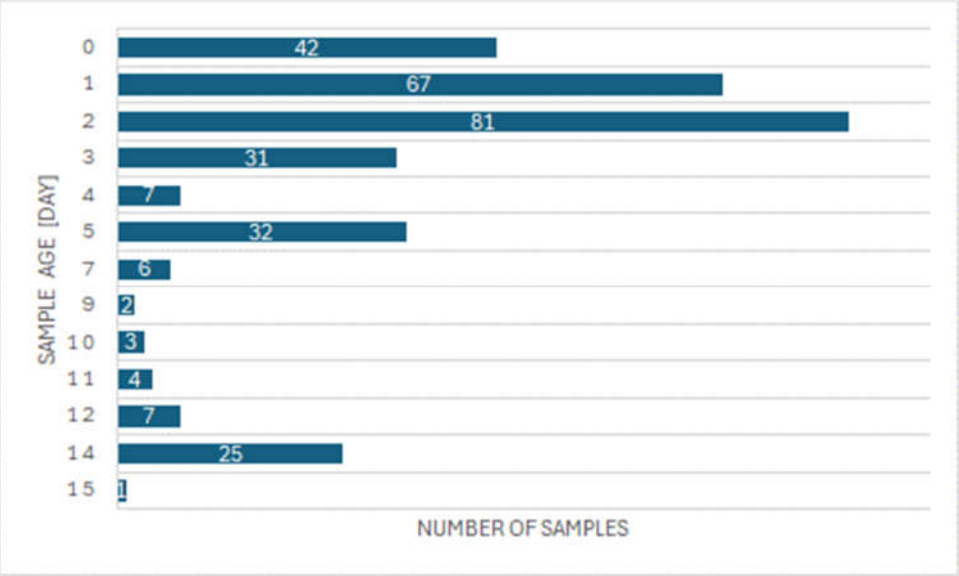

Figure 4: The age of collected noninvasive genetic samples, where sample age was recorded (n=308).

The experience from previous genetic monitoring of large carnivores, such as bear and wolf, have shown that subjectively estimated sample age is a good predictor of genotyping success. That is why this information should be recorded by the sampler. In bear scat samples where age is subjectively estimated, scats that were collected as 5 days have low genotyping success (33%), while genotyping success of fresh scats (0 days) is over 80% (Skrbinšek 2020). In such species where scats can be found relatively easy, the samples older than 5 days are not even collected. Because it is difficult to find lynx genetic samples, everything that was found was nevertheless collected and analyzed. With lynx samples, sample age was recorded for roughly less than half of collected noninvasive genetic samples (n=308). Out of these, where this information was available, 26% of samples were estimated to be 5 or more days old.

The differences in the genotypes of the Dinaric lynx are minimal and PIsib (probability of identity between siblings) is high. Some individuals are even homozygous on most of the loci, and we have seen how small genetic differences are between individuals when we sampled kittens in the same litter. The results of individual identification in Table 1 are summed up for 2019-2023 from the C5 reports, the time of the reinforcement of the population. Some of these individuals were detected over multiple monitoring seasons. Altogether in all genetic samples, analyzed within the project, we have identified 128 unique individuals.

All translocated lynxes were genotyped before release (actions C1 and C2). For each of them we tested relatedness with the lynx that had already been translocated and used this information when deciding on the release site. When compared with the allelic composition of the Dinaric lynx population, we could identify “private alleles” not present in the Dinaric population before the translocations. These alleles make translocated lynx and their offspring easy to detect, as the only way for an individual to have such an allele is through inheritance. Shown in Table 3 are the private alleles from all translocated lynx, also the ones that did not integrate in the population. There are 23 such private alleles on 12 loci, which makes detection of the first-generation offspring of the translocated individuals quite straightforward. The detected offspring were assigned to possible parents through simple exclusion. In this manner we genetically confirmed the parentage of five offspring of lynx Goru and remnant lynx Tea. In

the Alpine area we confirmed the parentage of offspring from Aida and Zois and Julija and Tris. Interestingly, only four offspring individuals were identified from noninvasive genetic samples, all others were identified from samples taken during capture for telemetry.

Table 3. Alleles found in 18 translocated lynx within LIFE Lynx and 2 translocated lynx within the UlyCa2 project that were not previously detected in the Dinaric lynx population. These "private" alleles make offspring of the translocated lynx very easy to detect.

Locus	Allele
F115	240, 244, 248, 252
Fca123	140
Fca132	175, 179
Fca001	181, 187, 191, 193
Fca650	129, 131
Fca161	184
Fca293	172
Fca424	168, 180
Fca247	151
Fca723	179, 191
HDZ700	141, 145
Fca201	141

Genetic structuring

Genetic structure was tested using principal component analysis (PCA) using the R statistical environment (R Development Core Team 2023) with the package 'adegenet' (Jombart, 2008). We visually examined the eigenvalues in a scree plot to determine the number of interpretable components and plotted the results to explore the patterns of genetic structure. In STRUCTURE (Prichard 2000) we examined the data with 100,000 burn-in and 1,000,000 MCMC repetitions, the admixture models and 3 population clusters (K=3) with five repetitions. The results for population clusters and individual assignments were then plotted with CLUMPAK (Kopelman et al., 2015).

Study areas in Romania and Slovakia are spatially relatively far from one another, on the opposite sides of the Carpathian Mountains. Certain levels of genetic structuring can be expected, but generally the lynx in Romania and Slovakia are a part of the same Carpathian population (Ratkiewicz et al 2012; Forster et al 2018).
As we can see in the result from the pre-reinforcement analysis of the genetic data (Skrbinšek et al, 2019), which also included historical lynx samples, a simple PCA clearly separates all three populations on two PCA axes (Figure 5). Dinaric population is differentiated from the

source population in Slovakia due to bottleneck and genetic drift. Three individuals sampled as "Dinaric" are close to the source population in Slovakia. One of these lynx is an old sample and may have been an old lynx born in some of the first generations after the reintroduction, and the other was an animal translocated from Switzerland to Italy in 2014. Another animal clustered half-way between the Slovak and Dinaric samples (this tissue sample was obtained anonymously in Croatia in 2016 and was later removed from downstream analysis, because reliable information on location and date were missing).

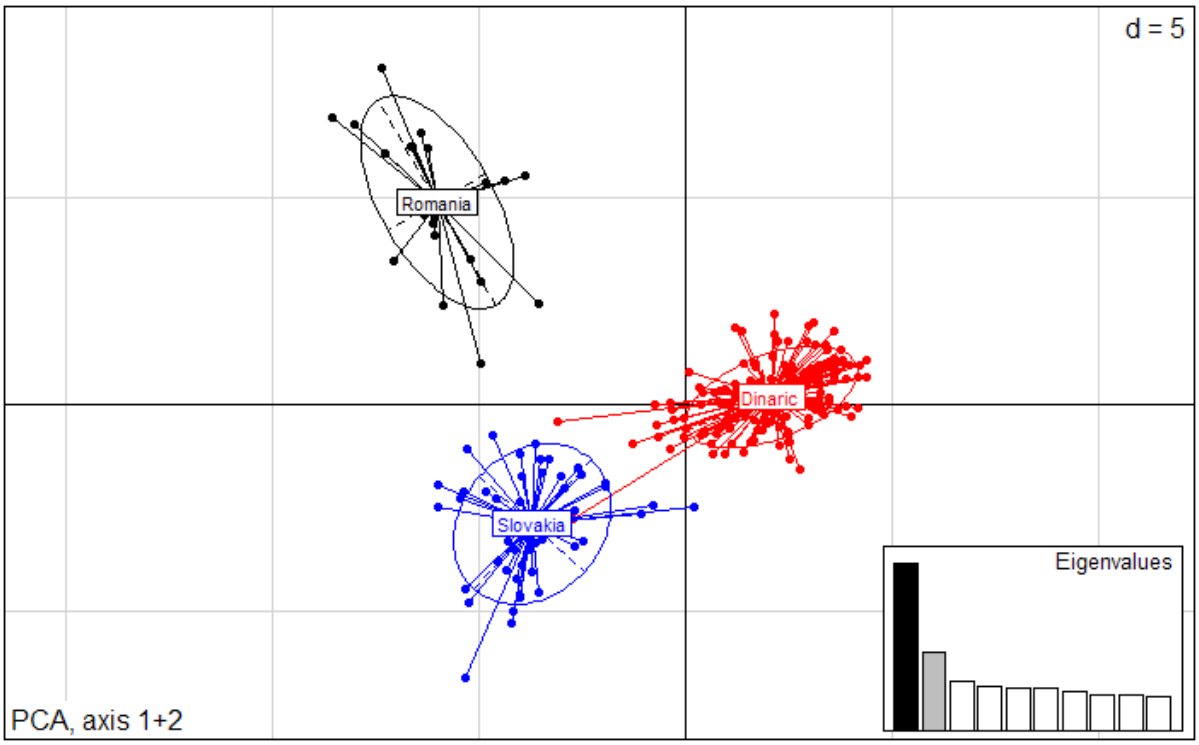

Figure 5: Principal component analysis of microsatellite data. Axes 1 and 2 (with high eigenvalues) show clear structuring both within the Carpathians as well as the structure caused by reintroduction sampling / genetic drift in Dinaric Mountains. Result from the A3 report baseline population study (Skrbinšek et al., 2019).

We updated the reference samples of lynx from Slovakia (n=38) and Romania (n=25), with samples collected after 2019 in each country, respectively. For Slovakia we excluded the samples from before 2010 to equalize the number of reference samples. Both analyses were repeated with new samples collected after the reinforcement took place in 2019. We included Reference samples from Slovakia (1), reference samples from Romania (2), translocated lynx from Romania (3), translocated from Slovakia (4), offspring of translocated lynx (5) and lynx from Dinarics genotyped after 2019 (6) (Figures 6 and 7).

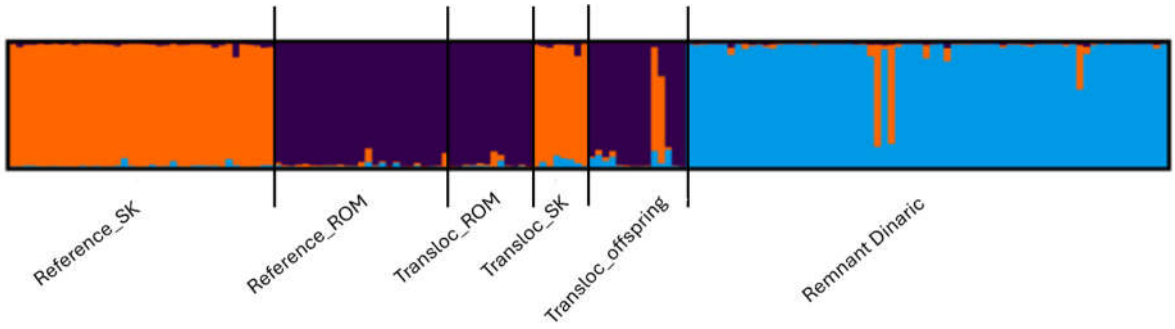

Figure 6: STRUCTURE analysis of microsatellite data, K=3. The translocated lynx cluster with their origin reference group as do their offspring. (1) reference samples from Slovakia, (2) reference samples from Romania, (3) translocated lynx from Romania, (4) translocated from Slovakia (4), (5) offspring of translocated lynx, (6) lynx from Dinarics genotyped after 2019.

We can clearly see genetic structuring among the three clusters, and the translocated lynx captured in Slovakia and Romania cluster with the respective reference samples. Remnant Dinaric lynx are genetically differentiated and in PCA closer to their original source population in Slovakia. However, there are two animals, the samples of which were collected in 2021, and they cluster together with the samples from Slovakia. We have excluded the option of them being the first-generation offspring from any of the translocated lynx. At the time only Maks and Doru were translocated, and a simple exclusion analysis of their genotypes shows that they can't possibly be the parents. These two animals also don't have any of the private alleles introduced through the translocations, so the most likely explanation is that they are clustering close to the Slovak lynx purely by chance. This is actually not surprising considering that the remnant lynx are descendants of a reintroduction from Slovakia, and that any genetic differences between both populations are purely because of genetic drift.

The only offspring of translocated lynx in the Dinaric area with available genotypes are from lynx Goru, who originates from Romania. The effect of "private" alleles clusters them with the Romanian population, although they are in fact admixed. The offspring from the Alpine area cluster with samples from Slovakia (offspring of Julia and Tris) or Romania (offspring of Aida and Zois). Lynx in the Julian Alps that are forming the stepping stone population are all either translocated lynx or their offspring, so genetically none of them cluster with the remnant lynx population. A genomic analysis of samples from Carpathians exhibits moderate levels of inbreeding in ROH (runs of homozygosity) analysis, which indicates moderate inbreeding in Carpathians (Mueller et al., 2022). This indicates that mixing of lynx individuals from Slovakia and Romania could be beneficial for fitness of the offspring.

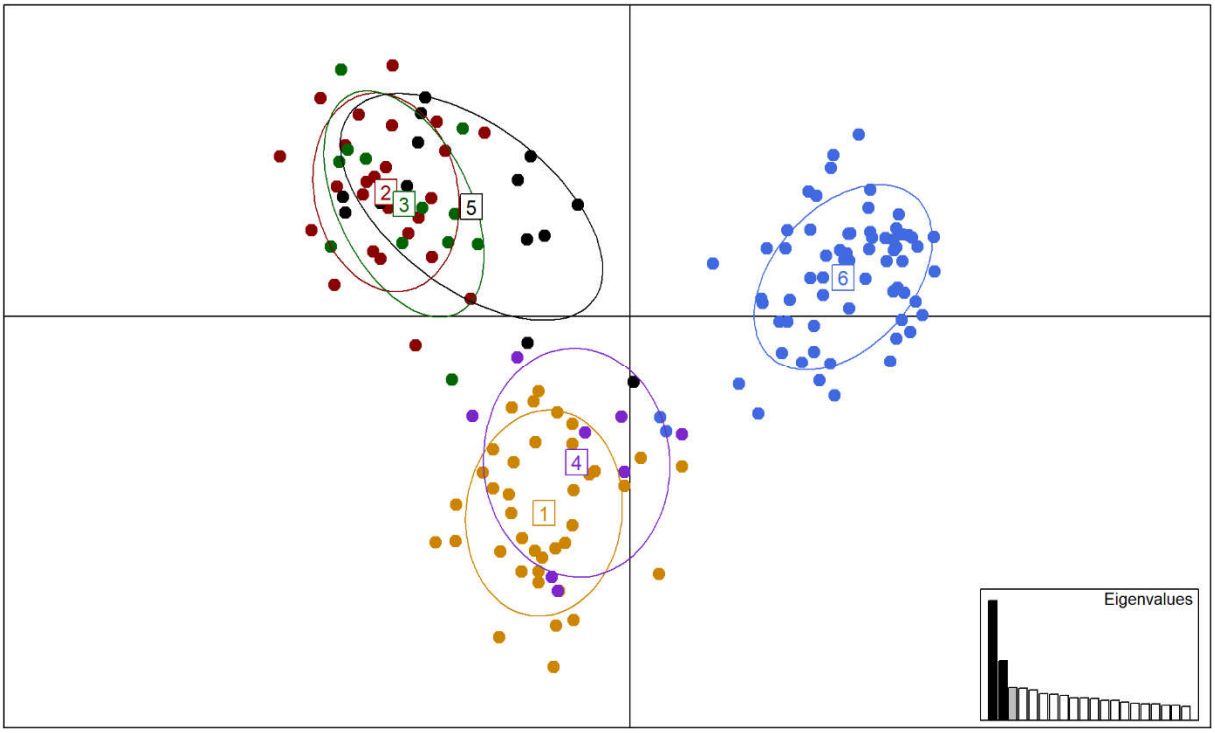

Figure 7: Principal component analysis of microsatellite data. Axes 1 and 2 (with high eigenvalues) show clear structuring both within the Carpathians as well as the structure caused by reintroduction sampling / genetic drift in Dinaric Mountains. The translocated lynx group with their origin reference group. (1) reference samples from Slovakia, (2) reference samples from Romania, (3) translocated lynx from Romania, (4) translocated from Slovakia, (5) offspring of translocated lynx, (6) lynx from Dinarics genotyped after 2019.

Inbreeding analysis

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 (R Development Core Team 2023) with the package 'adegenet' (Jombart, 2008).

Genetic diversity was measured as the observed heterozygosity (Ho) and Nei's unbiased heterozygosity (*He*) (Nei, 1978), using the R package 'adegenet' (Jombart, 2008). We used a traveling window analysis (Sindičić et al., 2013) to explore the erosion of genetic diversity caused by genetic drift in the Dinaric population, illustrating the dynamics of effective inbreeding through time. We used 40 samples as the width of the window.
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, because the population has been reintroduced from Slovakian Carpathians, the drift component of inbreeding (F_{st}) directly indicates the inbreeding of the Dinaric population relative to the source population in the Slovakian Carpathians. We used the term '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. We estimated expected heterozygosity of lynx in Slovakia at $He = 0.592$, using the same markers and 60 individuals (including the translocated animals). We used this reference to estimate the dynamics of inbreeding in the Dinaric lynx relative to the original source population in Slovakian Carpathians.
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 observed 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 $1 - \delta$.

In the collected (n=128) and historical samples (Sindičič et al 2013, n=90) as well as translocated animals (n=20) we identified 238 individuals, and we used genotypes of 228 individuals for downstream analyses. Some animals were excluded because of incomplete field data (missing year of collection, n=3), and we excluded the translocated animals that we know had died before reproduction or had no chance to reproduce (n=4).
As the Alpine stepping-stone subpopulation is currently in its first generation and hence completely outbred, it makes no sense to estimate inbreeding for that subpopulation. We explored three scenarios: including only lynx translocated to Dinaric Mountains and their offspring (isolated stepping stone scenario), and including all animals together with those in the Alpine stepping stone (fully connected stepping stone scenario). Figure 8 shows the dynamics of inbreeding under these three scenarios.

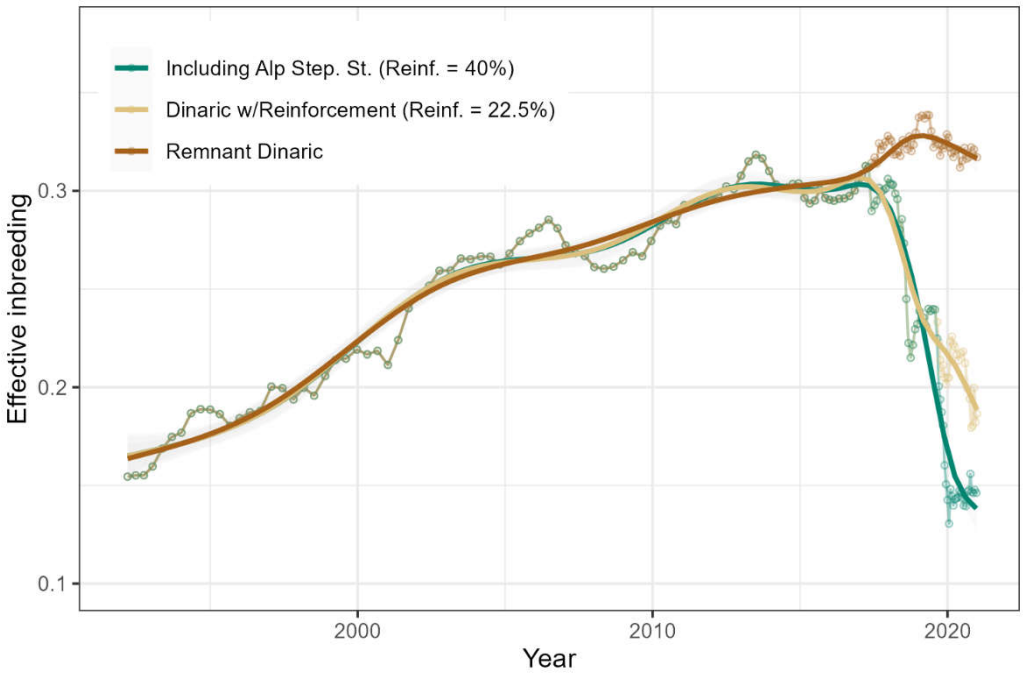

Figure 8: Effective inbreeding (Fe) of Dinaric lynx relative to the source population in the Slovak Carpathians, calculated using heterozygosity in the Slovak lynx (estimated from the Slovak samples) and a 40-sample traveling window. Remnant Dinaric: calculated without the translocated lynx and their offspring, exploring situation without the effect of translocations; Dinaric w/Reinforcement: only with lynx translocated to Dinaric Mountains and their offspring; Including Alp Step. St.: with all translocated lynx, including the Alpine stepping stone subpopulation. The "Reinf." value indicates the proportion of translocated animals and their offspring in the final traveling window.

As both the translocated lynx and their direct offspring are included in the estimates after 2019, we have a strong Wahlund effect (Wahlund 1928) in the final traveling windows. This is also clearly seen in genetic structure analysis (Figure 6). Using expected heterozygosity to estimate inbreeding, we can better understand the effect of the reinforcement on the genetics of the population using empirical data. According to the last period (last 40 most recently detected individuals in the dataset), in scenario without the reinforcement, inbreeding would remain high at around F=0.32, with inbreeding depression $\delta = 0.85$ (i.e. the fitness of these animals is expected to be 15% of the fitness in the source population). Considering the effects of translocations in the Dinaric mountains with translocated animals and their offspring representing 22.5% of the population (reflecting that ratio in the sample), inbreeding would be around F=0.19. Considering the recent SCR abundance estimates for the Dinaric part and the numbers of detected offspring (156 ± 19 adult lynx in 2022-2023, at least 52 kittens of translocated parents), we may already be beyond this value. We would expect inbreeding depression to be $\delta = 0.68$, and fitness more than double of its pre-reinforcement value at 32% of the source population fitness. If we include samples from the Alps (scenario of fully connected Alpine stepping-stone and Dinaric Mountains subpopulations) and with the translocated animals and their offspring forming a large part of the population (40% in the sample), inbreeding would drop to 0.08, and fitness increase to four times compared to pre-reinforcement (61% or the source population fitness).

Camera trapping

The transnational camera trapping served to obtain information about the distribution of lynx and the territorial pairs, the number of independent lynx, the sex ratio and the presence of reproductive units in the Dinaric Mountains and the SE Alps, and was described in detail in reports from the C.5 action. Specifically, the changes in the camera trapping effort and the demographic parameters over the years are thoroughly described in the final C.5 report (Fležar et al., 2024). As the data collected within C.5 action were processed and interpreted annually using the same approach, they are only partly comparable to the data collected within the A.3 action (Slijepčević et al. 2019), which is illustrating the first survey of the Dinaric - SE Alpine lynx population. Moreover, as the reintroductions began in 2019 with two animals (Doru, Goru) and the effect of that was limited to a single offspring detected until the end of 2020 survey (lynx Mala, the first offspring of lynx Goru), the status of the Dinaric-SE Alpine population in the first year of the C.5 action (2019-2020) still fairly represented the pre-reinforcement population status (see also Fležar et al. 2023). Finally, the quality of the camera trapping data collected within the C.5 action in the Slovenian and Croatian Dinaric Mountains (not including the Alpine area) was sufficient to be spatio-temporally adjusted to fit the requirements of the spatial capture recapture. That allowed us to assess the absolute population-level density and abundance estimates using SCR and infer about the population changes during the reinforcement process. Thus, this chapter is focused on the assessment of these changes using an adjusted camera trapping dataset collected within the C.5 action and analyzed under the SCR modeling framework.

Field design

Camera traps were set up exclusively in forested areas, which is the optimal habitat of lynx in the Dinaric Mountains (Skrbinšek and Krofel 2008). We classified the camera trapping sites into three distinct categories (hereafter 'location type'), based on their main characteristics; 1) lynx scent-marking site; i.e. forest cabins, conspicuous rocks, rock shelters, caves and other similar objects (Allen et al. 2017) confirmed to be used by lynx for scent-marking in this region by data collected through snow-tracking and/or camera trapping in the past or present survey (Slijepčević et al. 2019), 2) forest road; unpaved forest roads and logging trails and 3) other; animal trails, ridges, large rocks, rock walls and other prominent karstic landscape features but where no indication of lynx scent marking was observed (Table 4). We used camera-trapping sites with one or two cameras set, following the camera trapping guidelines from Stergar and Slijepčević (2017) to optimize lynx identification (see section 3. for further details). We used cameras with white flash (CuddeBack X-Change Color Model 1279, Cuddeback, Green Bay, Wisconsin) and Reconyx HyperFire 2 Professional White Flash Camera HP2W (Reconyx, Inc., Holmen) at sites where we expected lynx to pass by, and cameras of the same Cuddeback model with black (940 nm light wave) or regular infrared light (850 nm light wave) (StealthCam STC-G42NG, Stealth Cam, Irving; Moultrie M40-i, PRADCO Outdoor brands, Birmingham; LTL Acorn models Ltl-6310WMG and Ltl-6511WMC; Browning Spec Ops Elite HP4, Browning Trail Cameras) at sites where we expected lynx to stop for scent marking to avoid disturbing it with flash (Stergar and Slijepčević 2017).

Table 4. Camera trapping data per survey year, used for the SCR modeling shown as i) number of camera trapping sites set on marking sites, roads and other type of locations in the Dinaric Mountains, ii) cumulative number of and observed mean distance (km) between camera trapping sites (Nearest neighbor analysis tool, QGIS v. 3.6.0) per country (Slovenia, Croatia) and in total study area (Dinaric Mountains) and iii) the cumulative number of camera trapping days for the Dinaric Mountains.

		2019-2020	2020-2021	2021-2022	2022-2023
Number of camera trapping sites	**Marking site**	53	56	51	47
	Road	64	76	79	171
	Other	123	107	95	111
	Slovenia	146	131	129	119
	Croatia	94	108	96	210
	Dinaric Mts	240	239	225	329
Mean distance between camera trapping sites	**Slovenia**	1.08	1.29	1.40	1.47
	Croatia	2.34	2.25	2.52	1.80
	Dinaric	2.28	2.38	2.61	2.34
Number of camera trapping days	**Dinaric Mts**	24,874	28,142	29,069	34,817

In Slovenia, camera traps were set at all three types of locations, while camera traps were primarily set at marking sites in the Northern part of Croatia (Gorski Kotar), and at forest roads in the Southern part of Croatia (Velebit). Moreover, a higher density of camera trapping sites was set up in Slovenia compared to Croatia (mean 1.31 and 2.27 km between closest sites, respectively, see Table 4 for further details). To meet the minimum requirement of SCR analysis for ensuring a non-zero capture probability, we selected at least one camera trapping site in a potential home range of a studied population (Royle et al. 2014), the reported 95% MCP home-range size of a female lynx being just under 100 km2 (Hočevar et al. 2024). Moreover, even though camera trapping array was expanding over the years in Slovenia (Fležar et al. 2020, Krofel et al. 2021, Fležar et al. 2022, Fležar et al. 2023), we limited the camera trapping data for this survey to the sites located South of the A1 highway. The highway represents a serious barrier to the lynx home range, i.e. telemetry tracking showed no lynx established a territory overlapping the highway, but it rather confined to the South of the highway. Mean trap spacing and spatial scale parameters calculated by the models (see Results) confirmed that designs in all regions and the entire study area fit the standard recommendation of 2σ (Efford and Fewster 2013; Dupont et al. 2021).

For this study, we used data collected from August 15th to February 15th each survey year, yielding records from a total of 185 sampling occasions each year and 116,902 camera trapping days in total (Table 4), accounting for camera operability. Even though some camera trapping sites remained active after Feb 15th each survey year, we only considered data before that date to avoid potential bias in the spatial scale parameter as a consequence of changes in lynx

movement during the mating season and dispersal (Breitenmoser and Breitenmoser-Würsten 2008).

Data preparation

Lynx records were annotated with sex, age and individual identity. Sex was determined if genital area was clearly visible or if kittens were present with females, thus individuals were marked as females, males or unknown sex. Each record of lynx was subjected to identification based on the animal's unique pelage pattern (Topličanec et al. 2022) by trained observers (n=9), i.e. observers which have identified lynx during the previous (pilot) surveys, following the identification guidelines published by Choo et al. (2020). In case of poor-quality records, at least one additional observer attempted to identify the lynx independently until full consensus was reached, including annotating the individual as 'unidentified' if the observers could not agree on the identity of an animal. All records where consensus could not be reached and all records of lynx with only one flank of the body recorded were discarded from the analyses. Juveniles, i.e. kittens detected with their mother were also excluded from the analysis because of the high mortality of kittens (Andrén et al. 2006; Duľa et al. 2021). Thus, only data about individually identified independent lynx originating from high quality records where the pattern was clearly visible from both flanks of the body were used to build capture histories. We recorded the number of spatial recaptures in each region to check that our data met the recommendation of at least 20 spatial recaptures for accurate and precise estimation of the spatial scale parameter (Efford et al. 2004). Data about lynx and trap deployment were exported directly from Camelot software (Hendry and Mann 2017), which was used for camera trapping data annotation, and reorganized to fit SCR analysis.

SCR modelling

Lynx density, baseline detection rate and spatial scale parameter were estimated with maximum likelihood spatial capture-recapture models (Royle et al. 2014) using oSCR package (Sutherland et al. 2019) in R software v. 4.1.0 (R Core Team 2021). The distribution of individual activity centres was defined as Bernoulli random trials (Royle et al. 2014) and the spatial model of detection followed a half-normal detection function (Efford and Schofield 2020). We assumed homogeneous distribution of the individuals across space. We ran multi-session models with four sessions (1-4) defined as respective survey years (2019-2020, 2020-2021, 2021-2022, 2022-2023). At least 20 spatial recaptures were confirmed for each session and for the entire transboundary study area (Dinaric Mountains) (Efford et al. 2004). We first ran multi-session null models, followed by a set of models including local behavioural response ('b') to assess the individual-level variation in baseline detection rate at specific camera trapping sites (Royle et al. 2011), following the findings of Iosif et al. (2022). Next, we included the additive effect of session and sex on baseline detection rate and the spatial scale parameter, as suggested by Goldberg et al. (2015). Sex was included as a binary covariate (female as reference category) and session 1-4 as factorial covariate. Finally, we included the additive effect of location type as a categorical three-level (i.e., marking site, road and other) with marking site as reference category.

Table 5. Overview of lynx captures for the entire study area (Dinaric Mountains) per session. Each session corresponds to a survey period which lasted from Aug 15th to Feb 15th.

		2019-2020	2020-2021	2021-2022	2022-2023
Total spatial recaptures		84	119	109	170
Total recaptures		148	257	275	341
Mean recaptures		3.44	4.36	5.19	4.32
Mean spatial recaptures		1.95	2.02	2.06	2.15
MMDM		10.71	9.14	10.23	8.63
State space (km^2)		12,350	12,206	12,350	12,275
Number of individual lynx	*females*	19	26	21	27
	males	20	24	26	26
	unknown sex	4	9	6	26
	total	43	59	53	79

For each of the models, we defined the extent of the effective sampling area, i.e. the "state space", multiplying the respective σ values by 3 to buffer the camera trapping grid and using a resolution of buffer cells at most the full value of the same parameter (Royle et al. 2014). We set the buffer width to 15 km and the resolution of buffer cells to 2.5 x 2.5 km. We restricted the state space by excluding all cells located in non-lynx-habitat defined by the habitat suitability model for lynx in the Dinaric Mountains (Skrbinšek and Krofel 2008), i.e. the Adriatic sea, urbanized or highly agricultural land. We ranked candidate models based on Akaike Information Criterion (AIC), with models having $\Delta AIC \leq 2$ considered having substantial support (Burnham and Anderson 2004). Among the highest-ranking models, the one with the best fit (lowest AIC value) and the highest predictive power (highest AIC weight; Johnson and Omland 2004) was used to calculate the abundance of lynx at the level of the population, i.e. the Dinaric Mountains in Slovenia and Croatia.

Lynx abundance and density in the Dinaric mountains

In total, we detected lynx at 1021 independent occasions during the survey period, creating in total 234 individual capture histories (Table 5). One individual male lynx in Slovenia was recorded 19 times, while 14 lynx were recorded only once.
Out of 12 competing models, two of them were the top ranking models with the difference in AIC < 2. Thus, we choose the model with highest AIC weight and cumulative weight as the model with the most support.

The best model showed that the baseline detection rate differed between sessions, with the highest values being reached in the 2021-2022 survey year. Moreover, this parameter differed between female vs male lynx, as well as by the type of camera trap setting (marking site, road, other location). Specifically, the males were detected roughly twice as much by camera traps than the females, and the camera trap set up on lynx marking sites had an almost double, or triple, the chance of detecting lynx than roads, or other types of locations (ridges, stone walls, or similar features), respectively. It was also clear that the distance at which the females were detected from the center of their presumed home ranges was smaller (3.94 ±0.22 km) than males (4.98 ±0.16 km), i.e. indicating that the females have smaller home ranges, which is also confirmed by telemetry data presented in Hočevar et al., 2024.

Over the state space encompassing more than 12,000 km^2 each survey year, the lynx population density grew. In 2022-2023, it was estimated at 1.27 ±0.15 lynx / 100 km^2, which translates to 156 ±19 independent lynx. That is a 44.3 % increase in mean population density over the Dinaric Mountains in the course of four years, without any major changes in the area surveyed (only 6.5% larger state space).

Table 6. Mean female, male and total densities estimates over 4 survey years for a state space exceeding 12.000 km^2. Estimates are given with 95% confidence intervals (in brackets), while the precision of the estimate is illustrated with coefficient of variation.

	2019-2020	**2020-2021**	**2021-2022**	**2022-2023**
mean population density	0.88 (0.63-1.23)	1.05 (0.80-1.38)	0.91 (0.68-1.20)	1.27 (1.00-1.61)
mean female density	0.61 (0.42-0.88)	0.72 (0.52-1.00)	0.62 (0.45-0.86)	0.87 (0.65-1.16)
mean male density	0.28 (0.20-0.40)	0.33 (0.25-0.45)	0.28 (0.21-0.39)	0.40 (0.31-0.53)
mean population abundance	110 (79-152)	129 (98-169)	112 (84-149)	156 (123-198)
coefficient of variation	0.179	0.156	0.162	0.141

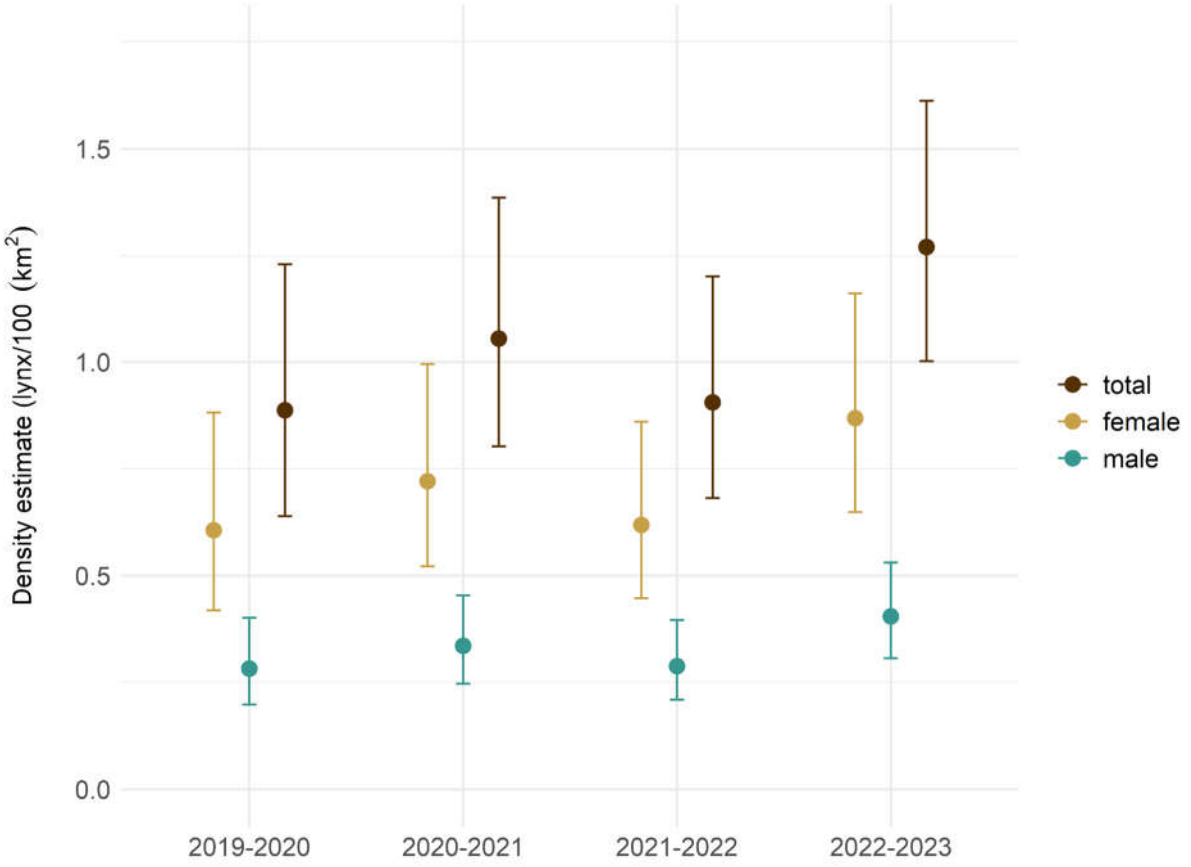

Figure 9. Density estimates for the lynx in the Dinaric Mountains; estimates for females, males and the total population are shown for each session.

Reproduction and distribution area

Among the 18 translocated lynx within the LIFE Lynx, we have detected mating or presence of kittens in the territory for 12 lynxes. Besides, two more have the potential to engage in reproduction in the future. Until 2024, we recorded in total 52 kittens with presumed or confirmed translocated parent(s) (Hočevar et al., 2024). This is likely an underestimation, because we did not include areas visited by the translocated males during their extra-territorial mating excursions (recorded in 10 out of the 15 mating seasons of the nine GPS-tracked translocated males).

We were able to confirm the parentage of translocated lynx in 10 out of 24 liters using genetic analyses. This is also the data we could use for estimating the inbreeding coefficient (Figure 8). The other conformation of parentage of the translocated lynx came from camera trapping data, where kittens with the rosette pattern were observed. The coat pattern is highly heritable (Kubala et al., 2020) and some of the translocated lynx do have the rosette pattern (Prostor et al., 2024, Predalič et al., 2024) and a female with kittens was observed in their territory.

In total, we obtained data from 97 lynx litters by camera-trapping between 2019 and 2023. When at least one of the parents was presumed to be a translocated lynx, litter size was 37%

larger compared to litter with both parents from the remnant population (Hočevar et al., 2024). The increase in litter size is indicative of a drop in inbreeding and an increase of fitness compared to the pre-reinforcement values. Another observation confirming the positive impact of the reinforcement of the Dinaric population is the higher survival probability in admixed offspring compared to remnant individuals (Hočevar et al., 2024).

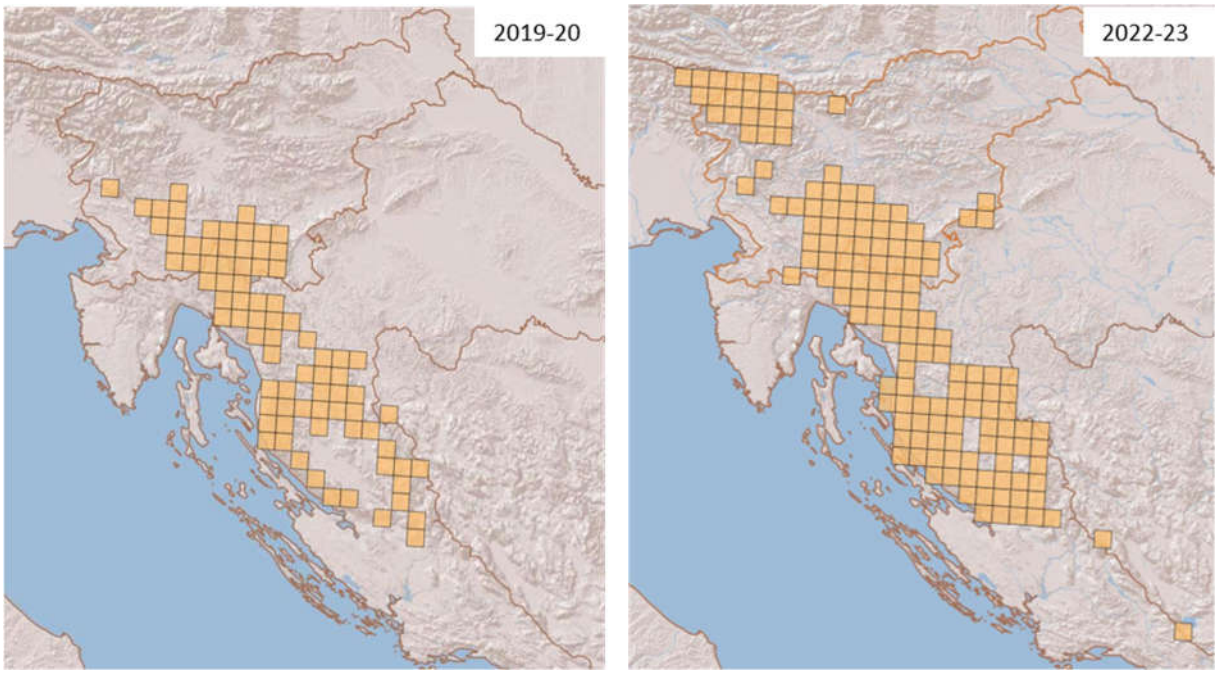

Figure 10. Lynx distribution in Dinaric-SE Alpine project area at the beginning and end of the reinforcement. Grid cells were colored on the basis of confirmed records of lynx in a standard European 10 × 10 km grid net. Four types of data were considered as confirmed lynx records: opportunistic data categorized as C1 or C2 record, GPS locations from collared animals with an established home range, camera trapping records and genetic records (Fležar et al., 2024).

Conclusions

As the Dinaric - southeastern Alpine lynx population was showing signs of being trapped in the extinction vortex and reached the level of inbreeding where immediate action had to be taken, the main goal of the LIFE Lynx project was to prevent its extinction. Successful translocation of Carpathian lynx and their integration in the remnant population represented the central part of the project.

We translocated 18 animals to Slovenia (12) and Croatia (6), with 12 lynx being released in Dinaric mountains and 6 lynx into the Slovenian Alps. Additionally, in scope of the ULyCA2 project, 4 additional lynx were released in the Italian Alps. Individual tracking of the released lynx showed high survival rates of translocated animals (86%). Initial high survival may be attributed to the use of wild-captured individuals, which was likely enhancing their hunting prowess (Hočevar et al., 2024). By the end of the project, the majority of the translocated lynx (54%) have also already successfully reproduced and integrated in the population (Hočevar et al., 2024; Fležar et al., 2024).

Understanding of the baseline genetic and demographic status of the reintroduced Dinaric lynx population (Skrbinšek et al., 2019; Slijepčević et al., 2019) and close surveillance of the reinforcement process (Fležar et al., 2024) allow us to assess the final effects of the reinforcement.

In the course of four years, we are observing 44% increase in the mean population density over the Dinaric Mountains, and an increase in the distribution area, especially through the creation of the stepping stone population in the SE Alps (Fležar et al., 2024). We are observing larger litter sizes in translocated lynx matings, and higher survival probability in admixed offspring compared to remnant individuals (Hočevar et al., 2024). Inbreeding dropped below the critical level, and corresponding fitness is expected to be more than double its pre-reinforcement value. These first, already detectable effects of the reinforcement, are confirming the project's success in preventing the extinction of the Dinaric – Southeastern Alpine lynx population.

In the genetic samples from the Dinaric mountains, translocated animals and their offspring represent 22.5% of the samples, and inferences about the genetic status of the population assume a similar proportion of such animals in the population. Inbreeding and genetic diversity in the population are currently difficult to assess precisely since it will require a couple of generations for allelic frequencies to stabilize (Luikart and Cornuet 1998), which means that our current estimates depend on the proportions of translocated animals and their offspring in the genetic samples. Since the abundance estimates for the Dinaric part and the numbers of detected offspring indicate that they represent a larger percentage of the population, the actual inbreeding may already be below the estimated value of F=0.19. These effects should become more prominent over the next few years as the translocated animals further spread their genes in the target population and allelic frequencies stabilize around Hardy - Weinberg equilibrium.

The stepping stone population in the SE Alps is still limited to an isolated area, and the effects of connecting the Alpine and Dinaric areas are yet to be seen. A few cases of lynx passing the Ljubljana-Koper highway have been detected (Kuralt et al., 2023). However, the computer modeling of the geneflow between the Alpine and Dinaric area indicates that even a low-level

geneflow between the two areas would significantly delay the inbreeding increase and make a considerable difference in the survival prospects of the entire Dinaric-SE Alps lynx population (Pazhenkova and Skrbinšek 2024).

If we include genetic samples from the Alps (scenario of fully connected Alpine stepping-stone and Dinaric Mountains subpopulations) in the inbreeding analysis, and with the translocated animals and their offspring forming a large part of the population (40% in the sample), inbreeding would drop to 0.08, and expected fitness increase to four times its pre-reinforcement value. Additionally, as phenotypic and genomic studies indicate moderate inbreeding in Carpathians (Kubala et al., 2020; Mueller et al., 2022), mixing of lynx individuals from Slovakia and Romania could be beneficial for fitness of the offspring and the vitality of the stepping stone population.

The main concern for the future viability of the lynx populations remains the isolation of the populations. Isolation with time leads to genetic diversity loss and inbreeding in all reintroduced populations (Mueller et al., 2022). The successful creation of a new stepping-stone subpopulation in the SE Alps therefore represents a major advance towards reaching the connectivity of lynx populations in the Alpine area (Hočevar et al., 2024, Potočnik et al., 2024, Pazhenkova and Skrbinšek 2024) and is of vital importance for lynx conservation in Europe.

But even with the success of this project, the population remains small and isolated. Dealing with this was planned in the project, and one of the key results of the project are the guidelines for ensuring the population's long-term viability (Pazhenkova and Skrbinšek 2024). Besides the future efforts to further connect the reintroduced lynx populations in a metapopulation, the population will require future support through translocations of outbred animals in regular intervals.

Final recommendations

In the beginning of the project we have prepared a detailed overview of past reintroductions (Wilson 2018) and took into account the experience to prepare a detailed population level reinforcement plan (Wilson 2019) as well as regional reinforcement plans (Pičulin et al., 2019, 2020, Sindičič et al., 2019). The activities in the donor population were carefully planned (Kubala et al., 2018a; Kubala et al., 2018b; LIFE Lynx project team 2018; Gazzola et al., 2018) and all project partners benefited from the knowledge exchange while preparing for the first lynx captures. During the project we have learned that when planning for a population reinforcement or long-term genetic management, selection of the optimal translocation strategy requires a careful consideration of several factors: translocation frequency, number of translocated individuals, genetic distance of the source population, source population dynamics, management and logistics (Pazhenkova et al., submitted). The baseline status of the Dinaric lynx population focusing on the status of the genetic and demography was assessed (Skrbinšek et al., 2019; Sljipčević et al., 2019; Fležar et al., 2019), which allowed us to evaluate the effects of the reinforcement.

We were able to maintain high public support for lynx conservation, while releasing predators, through intensive public communication campaigns and close partnership with the key stakeholders (Mavec et al., 2024; Velkavrh et al., 2024; Krofel et al., submitted).

Recommendations based on our experience are divided into three stages:

1. Establish the baseline status of the endangered population

It is important to understand the baseline status of the population being reinforced, with focus on genetics and demography. It is also important to analyze the samples from the source population(s), and to understand the status of the source population(s) well. This also allows for evaluation of the effects of removal of animals used for translocation since these activities shouldn't endanger the conservation status of the source (donor) population(s).

It is also recommended to genotype each animal that is being translocated and check for relatedness to other already translocated animals. This can be used as the basis for the decision on the release site of the individual to avoid releasing related individuals in the same or neighboring areas.

2. Implement a comprehensive transboundary monitoring program alongside the translocations

Wild-captured lynx were gradually translocated over the course of five years. The surveillance of the reinforcement process was conducted alongside the translocations, and the results obtained after each monitoring season served as guidance for the subsequent translocations in the next year. It is important to monitor the process both in the donor and the target populations. Focus on:

- Success of translocations - telemetry to monitor survival, movement, hunting abilities; camera trapping and genetic monitoring to detect translocated individuals and confirm reproduction.
- Impact on the population's demography - systematic camera trapping to estimate lynx abundance, detect reproduction events and estimate density of the population. Combination of several methods to monitor the area of occurrence.
- Impact on genetics - genetic monitoring to detect changes in genetic diversity and inbreeding levels.
- Ecosystem impacts - GPS tracking with field-checks of kill sites and analysis of GPS location clusters to monitor predation and scavenging.
- Maintaining public support - regular communication, direct stakeholder involvement, public attitude surveys.

3. Plan for maintaining of the long-term viability of the reinforced population:

Despite the successful reinforcement, the population can nevertheless remain small and isolated, which means that inbreeding will become a problem again if it's left to its own devices. The results of the monitoring and the final results of the project should be used to make a plan ensuring the population's long-term viability. In this project, the Guidelines for ensuring the long-term viability of the population (Pazhenkova and Skrbinšek 2024) were elaborated to provide guidance for maintaining the genetic diversity and avoid inbreeding depression in the future:

- Continuous monitoring of population development and the impact of the translocated animals - Regular genetic monitoring to detect early signs of declining genetic diversity, increasing inbreeding, or to detect habitat fragmentation. Regular camera trapping to monitor population size and density.
- Continuous efforts to connect the reinforced population with other nearby populations, with the long-term goal of creation of a meta-population.
- Use computer simulations to understand how the population's demography and genetics would change in the future. Use this as a basis for a long-term management plan.
- Implement a long-term strategy for genetic management of the population, ensuring enough geneflow to manage inbreeding, either through improving the population's natural connectivity, or through routine periodic translocations.

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