LIFE DINALP BEAR

Population level management and conservation of brown bears in northern Dinaric Mountains and the Alps

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GENETIC ESTIMATES OF CENSUS AND EFFECTIVE POPULATION SIZES OF BROWN BEARS IN NORTHERN DINARIC MOUNTAINS AND SOUTH-EASTERN ALPS

Report

Tomaž Skrbinšek, Maja Jelenčič, Roman Luštrik, Marjeta Konec, Barbara Boljte, Klemen Jerina, Rok Černe, Marko Jonozovič, Matej Bartol, Đuro Huber, Juraj Huber, Slaven Reljić, Ivan Kos

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The collaboration and the willingness to help of everyone included, as well as the international collaboration that developed and is still developing through LIFE DINALP BEAR and other initiatives, is something that transcends the daily ups and downs of relationships between people and countries. It is a thing to be cherished and nurtured in the years to come.

Executive Summary

Introduction

This report was produced as an output of the project action C.5, "Establishment and optimization of an integrated, population-level surveillance of brown bear conservation status". The overall goal was to establish robust, effective monitoring of brown bears in our project area. Genetics is one of the most important tools for this purpose, and probably the only tool that can give reliable answers to difficult but critical questions about a wildlife population – its size, dynamics, and long-term conservation outlooks.

The first part of this specific study was to determine, as precisely as possible, the **number of bears in NW Dinaric Mountains in Slovenia and Croatia**. We planned and executed an intensive genetic sampling of brown bears in their entire distribution range in both countries. We upgraded laboratory methods to increase speed of future analyses and decrease costs, providing solid foundations for long-term genetic monitoring of brown bears in the project area. And, we provided the first precise estimate of brown bear population size and sex structure in NW Dinaric Mountains in Slovenia and Croatia, setting a reference point for all future conservation and management of this species in our landscapes.

The second part of the study deals with more fundamental parameters – **genetic diversity and, importantly, effective population size**. Effective population size, or *Ne*, is one of the most important (if not the most important) parameters in evolutionary and conservation biology. While not completely accurate, it can be thought of as the number of animals that get to reproduce (usually quite different than the number of all animals in the population). This elegant concept describes in a single index both a population's sensitivity to genetic stochasticity (loss of genetic diversity and inbreeding) and summarizes the key information about population's evolutionary potential and probability of long-term survival. With this parameter estimated, we can start using the same population genetics theory to predict fate of populations in species with vastly different biology. The methodological and theoretical advances of the recent years are for the first time making feasible monitoring of this critical parameter in the wild.

Methods

Brief explanation of the principles applied in the study

We used noninvasive genetic sampling, next generation DNA sequencing and mark-recapture modelling to estimate the number of bears living in the NW Dinaric Mountains in Slovenia and Croatia. The idea is to collect noninvasive genetic samples (mostly scat samples, some hair and saliva samples) and through genotyping (producing genetic "fingerprints") recognize individual bears they originate from. In this manner we can directly count how many bears we've detected (the minimum number of bears in the population). Through capture-mark-recapture modelling we estimate the number of bears that we "missed" in sampling, thus estimating the total population size.

We also collected tissue samples of brown bear mortality and genotyped them with the final goal to determine effective population size of our bear population. In a finite population a non-random association develops between alleles on loci that are otherwise independent (linkage disequilibrium). The smaller the effective population size, the higher the linkage. Linkage disequilibrium between loci can be estimated from genetic samples, and used as a signal to estimate the effective population size.

Sampling and sampling success

We sampled the entire range of permanent bear presence in Slovenia and Croatia, covering over 20 000 km^2. The main part of the study area is in the Dinaric Mountains, which span the length of the Adriatic coast to form one of the largest continuous forest complexes in Europe.

Figure: Project areas of LIFE DINALP BEAR. Intensive noninvasive genetic sampling took place over the entire Dinaric Mts. project area, while opportunistic sampling (which is continuous throughout the project) was done in the Alps.

To manage sampling, we had to organize a large network of volunteers that would participate in sampling and provide samples. After consideration we decided to primarily target hunters and foresters (which are also among the most important interest groups in bear management). We tried to establish individual communication channels (through e-mail) with as many participants as possible, to establish individual-level participation and provide feedback. We provided volunteers with

"sampling kits" with material for sampling. Samples were delivered back to the laboratory through regular post using pre-addressed, postage paid envelopes included with sampling kits.

We monitored the sampling effort in real-time. All samples were entered in a cloud-based geo-database immediately when they arrived to the laboratory. Geo-database automatically plotted the samples on maps, enabling us to constantly track the progress of sample collection. We regularly checked the maps to see if there are any "blank areas" where samples were not being collected. Keeping the track of sampling as it was progressing allowed us to address these issues before they would become a problem.

Laboratory analyses, numbers of processed samples and genotyping success.

DNA in noninvasive genetic samples is typically of very low quality and quantity, and contamination is a serious issue that needs to be handled carefully. We used a dedicated laboratory for noninvasive genetic samples for DNA extraction and PCR setup, and followed very strict contamination prevention protocols. We tracked each sample using 2D barcodes, eliminating manual entry of sample ID codes and cataloguing each critical step from the entry of the sample into the lab until the final genotype.

We started using laboratory automatics (liquid handling robot) for DNA extraction and some critical pipetting steps to increase throughput and reliability. For genotyping we used a recently developed method that taps the power of next generation (high-throughput) sequencing (NGS), and solves many problems that plagued the "standard" approaches (difficulty to compare results between laboratories, subjectivity in genotyping…), increases genotyping success, and considerably speeds up analyses while lowering the costs (De Barba et al., 2017). We're collaborating with Laboratoire d'Ecologie Alpine (LECA) from Grenoble, France, which developed the method. As far as we know we are currently the first laboratory implementing this approach in a large real-world study.

With the NGS protocol we used 13 microsatellite markers + sex ID marker for individual ID genotyping of noninvasive genetic samples. For tissues, we extended this with another 16 microsatellite markers for a total panel of 29 markers for population genetics studies.

Data analysis

All data about samples and genotypes is kept in the MisBase database application developed in our laboratory, which we also use for some critical data manipulation and analysis steps, particularly matching of samples (identifying which samples belong to which bears) and genotype reliability assessment. To analyse the NGS microsatellite data we used some of the tools provided by the method's authors, but also programmed our own tools that we needed for the large-scale analyses.

To estimate census population size, we used capture-mark-recapture (CMR) modelling. We used several approaches (Capwire, MhChao and Huggins models) and constructed a very large number of differently parametrized models in an attempt to include all peculiarities in the data. We selected the most appropriate models to produce the final results, but we're also reporting the results of the competing models in the full report.

Sex ratio was estimated both from modelling results as well as directly from the detected animals. While results of all methods were nearly identical, the second method is simpler, has less assumptions and was used for the final sex ratio estimates.

With the tissue samples, we looked at some basic population genetics and genetic diversity parameters using R statistical environment with specialized packages for population genetics, and did a quality check of the data to identify any data quality problems. We did a quick PCA analysis of the genotypes to see if there are any clear indications of population structure or immigrants in the population.

We used the linkage disequilibrium method (LD) to estimate effective population size (*Ne*) and how it changed through time. Since the majority of bears included in the study through mortality sampling had age determined from tooth dentine layers, we used this age data and the year of death to determine when animals were born. We then created yearly samples for each year from 1997 until 2014 that included all animals aged 0 to 8 years (generation interval) alive in a target year. We used these yearly samples and program NeEstimator to produce reasonably unbiased estimates of *Ne* for each year in the studied period. For each year we made three estimates, using all samples and using samples for each respective country.

Results

Sample collection

In the period between September and December 2015 we managed to collect 4687 noninvasive genetic samples, 56 % more than the target 3000 we planned during project preparation. Spatial and temporal coverage were in most cases good. We received samples from 962 people, and we estimate that over 2500 people were actively participating in the study (not all of them were able to find samples, also because many are in the areas where bears occur sporadically). We feel that the action was a complete success.

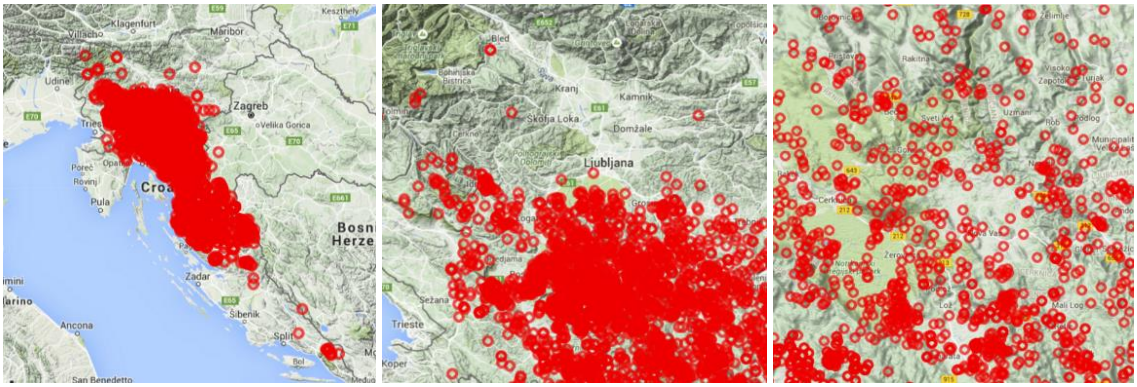

Figure: Collected noninvasive samples (total area and 2 zoomed-in views) viewed in the online sampling monitoring system.

In parallel we're routinely collecting genetic samples of dead bears in both countries. Each detected bear mortality is sampled, weighted, measured, and a tooth is taken for age determination. We have in genetic bank currently tissue samples of 2468 individual bears, collected over the span of the last 17 years.

Upgrade of laboratory methodology

As the final goal of Action C.5 was to establish long-term monitoring of the bear population, we worked to provide a solid foundation for that by improving our analytical pipeline for genetic samples. With use of laboratory robotics and the switch to next-generation sequencing (NGS) for genotyping v

we considerably decreased the manual workload in DNA extraction and allele calling (which was mostly done semi-manually before, but is now handled by bioinformatics). This resulted in a much shorter analysis turnover time, and a decrease in costs. Both ultimately made possible analysis of a considerably larger number of samples than what we originally planned in the project. The advantages of the methodology improvements are summarized in the table below.

Table 1: Performance comparison of the 'standard' genotyping methods we used before upgrading, and the 'new' methods used to analyse samples in this study. Throughput estimates are all based on a single person performance, with some non-expert help (in our laboratory usually students) in sample preparation for DNA extraction.

Item	Previous pipeline	New pipeline
DNA extraction	Manual, spin-column based extraction, **23 samples/day + 1 NC**	Automated DNA extraction with a liquid handling robot, **95 samples/day + 1 NC*.** *Can be doubled with a relatively modest investment.
Genotyping	Capillary electrophoresis & computer-assisted allele calling, **30-40 samples/day**.	Next generation sequencing and automatic allele calling from sequences, **300-400 samples/day**.* *Can still be increased several fold with a relatively modest investment.
Transferability of data	Subjective, laboratory-specific allele calling, **poor transferability** of the data.	Objective, sequence-based allele calling, **complete transferability** of the data.
Future-proofing for longitudinal studies	Data problematic for longitudinal and time series studies even within a single laboratory (changes in equipment and personnel can introduce inconsistencies in the data).	Data at the most basic level, the DNA sequence. Completely future proof, just as useful 100 years from now as it is in this moment.

Genotyping of noninvasive genetic samples

Although we originally planned to process 3000 samples of the intensive genetic sampling in Slovenia and Croatia, the advances in analytical procedures allowed us to process **4370 samples**, 93% of the collected samples and **45.7% more than planned**. This alone shows the benefits of the new methods for large-scale genetic monitoring of wildlife. The remaining samples are relatively old (lower expected success rate), and are all in Slovenia where sampling was much more intensive and additional samples would have negligible effect on results.

We managed to successfully genotype noninvasive **3218 samples**, or **73.6%** of processed samples.

Genotyping of tissue samples of detected bear mortality

Parallel to noninvasive samples genotyped within the scope of the intensive 2015 sampling, we also genotyped all detected bear mortality during that period, 142 individuals, and included the genotypes in the dataset for mark-recapture estimate of brown bear abundance.

Tissue samples are routinely collected both in Slovenia and Croatia. The total bear mortality dataset currently includes genotyped samples of **2022 individual bears** (1040 from Slovenia and 982 from Croatia). We've been routinely using a 16-locus panel + a sex-id marker for genotyping using capillary electrophoresis. We re-analyzed all samples collected prior to this project (1326 samples) with the new NGS markers, both to ensure compatibility with the noninvasive dataset and to increase the information content for downstream analyses. This brings the total dataset to **29 polymorphic microsatellite loci** + 2 loci for confirmation of field determined sex id. There are still 446 tissue samples in the process of being genotyped at the time of writing of this report. Since bear mortality continues to be sampled, we will analyse new tissue samples throughout the project. However, this is already one of the largest brown bear datasets in the world, and more than adequate fort the purpose of estimating temporal variation in effective population size reported here.

Population size estimates and sex ratio for the entire study area and individual countries

After inclusion of tissue samples of mortality during sampling and filtering a few problematic samples (data recording problems, genotyping issues) we ended up with 3263 samples that should be error free and suitable for capture-mark-recapture (CMR) analysis. Results are summarized in the table below.

Table: Genotyped samples, numbers of captured animals and mortality.

Samples and captured animals	Entire Area	Slovenia	Croatia
All genotyped samples*	3263	1962	1539
Total captured animals*	1136	614	582
Females	669	366	339
Males	467	248	243
Total recaptured animals	730 (64.3 %)	427 (69.5 %)	361 (62 %)
Total transboundary recaptured animals		14.10%	16.60%
Mortality			
Total mortality during sampling	142	65	77
Females	63	27	36
Males	79	38	41
Transboundary – died in the neighboring country		5 (2F, 3M)	2 (1F, 1M)
Total mortality in 2015	256	112 (67M, 45F)	144 (92M, 50F, 2 unknown sex)

*The totals are correct, but the animals crossing the national border were counted in both countries.

Spatial distribution of samples was good (see figure below), with the exception of Eastern Lika area in Croatia where intensity of sampling was lower, but still acceptable. Sampling intensity, and with it capture probability, varied considerably between areas. With MARK models (Huggins) we included this in models, but with the other models this is not possible so we made separate model sets for each area and combined the results, correcting for animals that crossed between the areas. Different modelling approaches provided very similar results (by-model results are in the full report below).

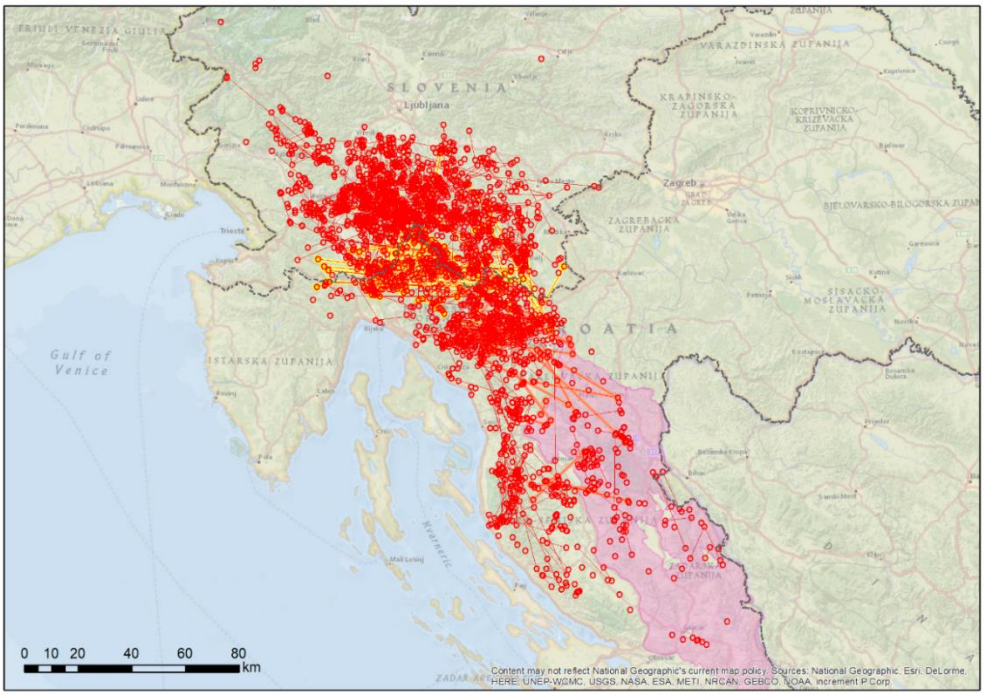

Figure: Successfully genotyped samples. Lines connect samples of the same animal (paths), paths of transboundary animals between Slovenia and Croatia are marked yellow. The area of Eastern Lika where sampling intensity was lower is marked in pale red, and paths of animals crossing in and out of that area outlined orange.

We estimated two parameters:

The minimum yearly abundance was estimated directly through mark-recapture analysis, which was done at the end of the year. The mortality detected during the sampling was subtracted (or not included, depending on the model) from the mark-recapture estimate to obtain the final value. This is the end-of-the-year estimate for 2015, after mortality and before next reproduction.

The maximum yearly abundance is derived from the minimum yearly abundance through addition of all detected mortality in that year. In our case it means the estimated abundance of brown bears in spring 2015. It is an underestimate since it doesn't take into account undetected mortality, which is assumed to be relatively low in brown bears (with the possible exception of the cubs of the year which may go undetected).

Table: Noninvasive genetic sampling and mark-recapture modelling estimates of minimum and maximum brown bear abundance and sex ratio in NW Dinaric Mts. in 2015.

Area	CMR Model	Minimum Yearly N (95% CI)	Maximum Yearly N (95% CI)	Sex ratio F:M [%]
Entire study	MhChao+Capwire TIRM	1392 (1247-1583)	1648 (1503-1839)	58.9 % : 41.1 %
Slovenia	MhChao	599 (545-655)	711 (657-767)	59.6 % : 40.4 %
Croatia	MhChao+Capwire TIRM	793 (702-928)	937 (846-1072)	58.2 % : 41.8 %

Population dynamics and management considerations of the genetic mark-recapture population size estimates

For Croatia, this is the first reliable estimate of brown bear abundance, while in Slovenia a similar study was already done in 2007. The 2007 the population size for Slovenia, the minimum yearly population size corrected for edge effect of transboundary animals shared with Croatia, was estimated 424 (383-458) bears. The methodologically very similar estimate for 2015 obtained in this study is 599 (545-655) bears, or a 41.3 % increase over the period of 8 years.

Ours is the first estimate of the brown bear abundance and sex ratio in NW Dinaric Mts. based on hard empirical data, setting the first reference point for any future studies. As such it represents the best possible foundation for science based conservation and management of this species in the entire area.

At the level of the entire study area this is still a "snapshot" of the situation, but in Slovenia, where a similar study was already done in 2007, it is already genetic monitoring with the temporal component. With it we have the first direct estimate of population dynamics in the area - it looks positive for bear conservation, but is also opening a wider discussion about tolerance of bears, and about future of bear management and conservation. Still, whatever the final decisions will be, they will have the best possible science to lean on - should the decision makers so choose.

Genetic diversity and population structure of brown bears in NW Dinaric Mts.

Since genetic diversity of brown bears in the study area has already been researched this was not the main goal of the study. Still, since a much larger locus set and more samples were used in this study and since genetic diversity indices are an important indicator of a population's genetic "health", we looked into those.

Two of the markers show deviations from Hardy-Weinberg proportions (most likely null alleles or genotyping errors) and were excluded from the downstream analyses. For the remaining 27 loci and all samples (N=2021) expected heterozygosity is 0.72, observed heterozygosity 0.71 and average allelic diversity 8 alleles per locus.

While we didn't detect obvious genetic structure, we did detect one individual with an unusual genotype. It's too early to declare it's an immigrant from another bear population, but we're researching this further. This animal was excluded from other analyses.

Temporal variation in effective population size of brown bears in NW Dinaric Mts.

Large number of samples and age data allowed us to estimate effective population size (*Ne*) of the studied brown bear population for the 18-year period from 1997 until 2014. For this period we were able to construct large enough samples of animals aged 0-8 years (~ generation interval) to provide comparable estimates of *Ne* for each year. The estimate is poor for Croatia for years between 1997 and 2001 where the number of samples from that area is small, as is evident from confidence intervals. After the year 2014 we don't have adequate sample coverage in all cohorts to provide reliable results. The most recent effective population size estimate for 2014 is *Ne* = 261.6, with 95% confidence interval 247.5 – 277.0.

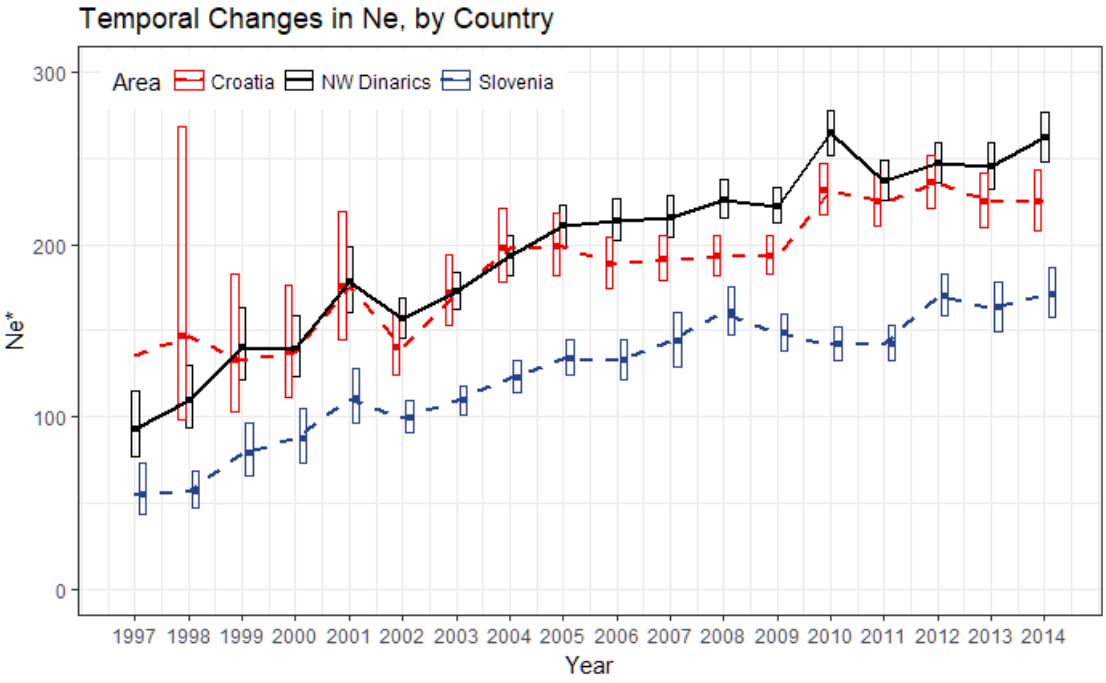

Figure: Temporal variation in effective population size of brown bears in NW Dinaric Mts. from 1999 until 2014. Estimates are made using all samples (NW Dinarics) and samples from each respective country. Animals aged 0 to 8 years (~ generation interval) and alive in the target year were used in each yearly sample. The estimates are not independent, but each estimate in year *t* represents the harmonic mean of *Ne* for the period *t* - GI, where GI is generation interval ~ 8 years.

We see that total effective population size is growing through time, and seems to have more than doubled since the end of 1990s. This is probably the consequence of increasing census population of the brown bear population in this area over the last two decades. The rate of this increase seems to be slowing down (but is still positive) in the period after 2005, which could be the result of the considerably increased culling in both countries after 2002.

An interesting result is that estimates from each country and both countries together provide different estimates of *Ne* (Figure 20). While estimates using samples from both countries and only samples from Croatia match quite well, the estimates using Slovenian samples are consistently and considerably lower. This result is surprising – if animals from both countries were mixing in the same population, as they should according to telemetry data, all these estimates should be approximately the same.

In the same time we see a considerable difference in management approaches. In Croatia the system was essentially trophy hunting, with most animals that were killed being adult males. In Slovenia culling is strictly regulated and targets mostly young (pre-reproductive) animals. This causes considerable differences in sex and age structure of the cull (analysis shown in the full report).

In Slovenia, high mortality of young animals and low mortality of adults limits recruitment into reproductive classes. This causes the same few animals to monopolize reproduction, decreasing the pool of parents, increasing variance in lifetime family size (most animals have no offspring, few animals have a lot of offspring) and decreasing effective population size. Also, high mortality in pre-reproductive classes and young animals makes any immigrants from Croatia less likely to succeed, effectively limiting geneflow from Croatia and “localizing” genes of Slovenian bears.

x

In Croatia on the other hand the trophy hunting approach to bear management causes quite the opposite effect. High mortality of adult males "makes room" for young males to join reproduction. Young females likewise have low mortality, and once they join reproduction they are largely protected when they have young with them. This decreases lifetime variance in family size and increases effective population size. An interesting effect can be expected on geneflow from Slovenia, since young animals from Slovenia have a higher chance of surviving and joining reproduction in Croatia than in Slovenia, causing geneflow into Croatia and "mixing" the genes.

Effective population size – conservation and management considerations

Effective population size of the brown bear population in NW Dinaric Mts. is not small (the 2014 estimate is 261.6 (247-277)). This is enough to avoid inbreeding, but still shy of the rule-of-the-thumb 500 threshold considered important for preserving evolutionary potential. This certainly emphasizes the importance of allowing and promoting connectivity towards other bear populations in the wider region, but doesn't necessarily mean that effective population size in NW Dinaric Mountains should be increased by other management changes as a matter-of-fact.

We can see that the different management models applied by Slovenia and Croatia over the relatively small and well-connected area of NW Dinaric Mountains have considerable and rather unexpected effects on the most basic genetic parameters critical for conservation. It's been known and discussed for quite a while that humans can have considerable effect on evolution of species they interact with, which may be particularly true for the wildlife species that are harvested or otherwise managed by lethal means. This is something that will need to be discussed in the future for our bears, and taken into account in management planning. Both management models have their pros and cons (further discussion in the full report), and a wider debate should be started about how to include this new understanding into practical bear conservation and management.

Contents

Introduction

This report was produced as an output of the project action C.5, "Establishment and optimization of an integrated, population-level surveillance of brown bear conservation status". As the name of the action says, the overall goal is to establish robust, effective monitoring of brown bears in our project area. Genetics is one of the most important tools for this purpose, and probably the only tool that can give reliable answers to difficult but critical questions about a wildlife population – the questions of its size, dynamics and long-term conservation outlooks.

The first part of this specific study was to determine, as precisely as possible, the number of bears in NW Dinaric Mountains in Slovenia and Croatia. The best method we currently have for such studies is noninvasive genetic sampling, paired with mark-recapture modelling. In the study we planned and executed an intensive genetic sampling of brown bears in their entire distribution range in both countries. We upgraded laboratory methods to increase speed of future analyses and decrease costs, providing solid foundations for long-term genetic monitoring of brown bears in the project area. And, last but not least, we provided the first precise estimate of brown bear population size and sex structure in NW Dinaric Mountains in Slovenia and Croatia, setting a reference point for all future conservation and management of this species in our landscapes.

The second part of the study deals with more fundamental parameters – genetic diversity and, importantly, effective population size. While maybe a bit esoteric to the uninitiated, effective population size, or *Ne*, is one of the most important (if not the most important) parameters in evolutionary and conservation biology. This elegant concept describes in a single index both a population's sensitivity to genetic stochasticity (loss of genetic diversity and inbreeding) and summarizes the key information about population's evolutionary potential and probability for long-term survival. While notoriously difficult to estimate reliably and often misunderstood in the past, the methodological and theoretical advances of the recent years made monitoring of this critical parameter in the wild feasible. We may be some of the first to start doing this routinely for a non-model species.

The study was difficult and required enormous amounts of work both by project personnel as well as by the huge number of volunteers. However, the final results are excellent, and we're proud of the achievement.

Methods

We used noninvasive genetic sampling, next generation DNA sequencing and mark-recapture modelling to estimate the number of bears living in the NW Dinaric Mountains in Slovenia and Croatia. We also collected tissue samples of brown bear mortality and genotyped them with the final goal to determine effective population size of our bear population.

The methods are state of the art, and for some of them we may be the first to use them in a large, real-world study. While we'll try to describe them in enough detail for the purpose of the report, we will also list appropriate references that will enable an interested reader to further research and understand them in detail.

Study area

We sampled the entire range of permanent bear presence in Slovenia and Croatia, covering over 20 000 km^2 (Figure 1). The main part of the study area is in the Dinaric Mountains, which span the length of the Adriatic coast to form one of the largest continuous forest complexes in Europe. Density of human population is relatively low for European standards. Human residence is in most cases limited to valleys, leaving large, continuous patches of dense forests that expand across the border between both countries for wildlife. The most common forest plant community is the Dinaric beech-fir forest, *Omphalodo-Fagetum*. A part of the study area to the south belongs to the Mediterranean biogeographic region, and the western part assumes pre-Alpine characteristics. While there are bears present in the Julian Alps in the Alps project area (see Figure 1), these are just few individuals and intensively sampling these areas wouldn't have an effect on the total population size estimate. We did sample these areas opportunistically to follow bear expansion into the Alps (which is another project goal), but the main goal in this study was to genetically "tag" the bears there, with mark-recapture population estimate being secondary to that.

Figure 1: Project areas of LIFE DINALP BEAR. Intensive noninvasive genetic sampling took place over the entire Dinaric Mts. project area, while opportunistic sampling (which is continuous throughout the project) was done in the Alps.

Abundance estimates - study design and power analysis

In previous studies using non-invasive genetic sampling in Slovenia in years 2003-2008 we obtained the highest amplification success rate from scat samples in autumn and early winter (Tomaž Skrbinšek, Jelenčič, Waits, Kos, & Trontelj, 2010; Tomaž Skrbinšek, Potočnik, Kos, & Trontelj, 2007). In the same studies we also found that the population behaved as an approximately demographically closed even in small study areas if samples were collected within a three-month timeframe (Tomaž Skrbinšek et al., 2007). This experience was successfully used in the previous intensive genetic sampling in Slovenia (Tomaž Skrbinšek, Jelenčič, Potočnik, Trontelj, & Kos, 2008), and was also the basis for the decision to plan for an intensive, three month long non-invasive sampling session in autumn/winter 2015, from September 1 until the end of December.

To understand the sampling effort required to obtain a reasonable confidence interval of the mark-recapture estimate, we performed a power analysis using a simulation study in program MARK (White & Burnham, 1999). This was done already in the project preparation phase. We used 2000 animals as the best-guess upper limit population size, a pessimistic 70 % expected genotyping success rate and a simple p=c(.) Huggins' model (Huggins, 1989) as both the simulation and the estimation model. We simulated different sampling intensities to understand the width of confidence interval that would be obtained in the ideal circumstances. We used the results to plan sample collection (the goal was 3000 samples in both countries) and scale the sampling effort.

Motivating and managing a network of volunteers, monitoring of sampling effort and providing feedback

Organizing volunteer network

Since sampling was planned over a very large area in a very short time, it was practically impossible to do it with project personnel. To manage it, we had to organize a large network of volunteers that would participate in sampling and provide samples.

After consideration we decided to primarily target hunters and foresters (which are also among the most important interest groups in bear management) and not to explicitly target the general public. With this in mind, our main promotional/recruiting activities were planned for the main target groups. To reach out to hunters, we published articles in the main hunting magazines in Slovenia and Croatia, explaining the study and asking them to participate. We also contacted the national hunting organizations in both countries, as well as individual hunting clubs throughout the area. In late spring and summer 2015 we organized meetings with representatives of hunting clubs through the entire study area. We organized meetings in Slovenia (8 meetings) and in Croatia (48 meetings). Because of the differences in organization of hunting, organizing meetings in Slovenia was easier, and consequently less meetings were needed to cover the entire bear range. We introduced the action to the representatives of hunting clubs, asked for collaboration and provided each of them with sampling material for their hunting club. To obtain as consistent sampling effort as possible, we adjusted the number of sampling kits for each hunting club with regard to the area of forest they covered (obtained thorough GIS analysis). To economize, we adjusted the number of sampling flasks in each sampling kit in accordance with previous information about expected bear densities (i.e. in core areas we provided 5 tubes per kit, in the edge areas with sporadic bear presence we provided 2 tubes per kit).

Foresters, on the other hand, were contacted and asked for participation through the national forestry services (Slovenian Forest Service, Croatian Forest Service), and we used these organizations' internal structures to distribute sampling material to their workers. With exception of the hunting areas that are managed by forest services, they provided a parallel sampling network covering also the area covered by non-professional hunters. The hunting areas managed by the national forest services were sampled by professional hunters employed there.

Contingency planning

As we planned to do real-time monitoring of the sampling effort, we also planned for contingencies that would allow us to rapidly react to any detected problems. The scheme is outlined in Figure 2, but the main idea was that if any area was being undersampled for whatever reason, we would first a) contact local resources to see what the problem is and b) failing to find a solution, organize sample collection in these areas either using alternative local resources or project staff.

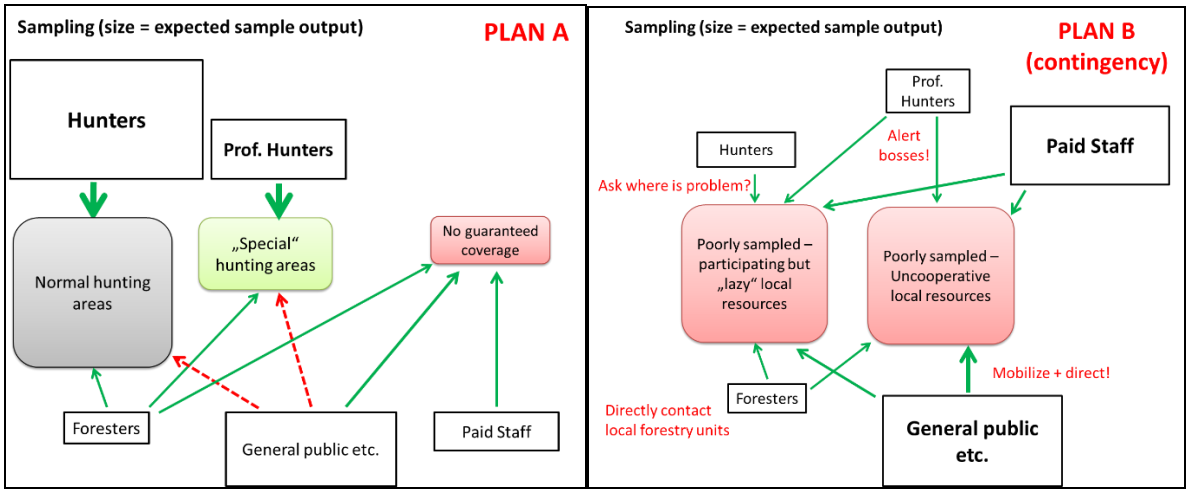

Figure 2: Planned sampling by different groups (Plan A) and contingency plan when something went wrong (Plan B).

Sampling material, communication with volunteers, sample collection and returning of samples to the laboratory

We designed sampling materials and an instruction brochure into personal “sampling kits” (Figure 3) The main philosophy was to make sample collection as easy as possible for all participants – everything they needed for sampling was included in the kit, they just needed to collect the sample when they found one. The kit included a detailed instructions brochure in the local language and sampling tubes in resealable plastic bags with a printed paper label containing a data-entry form to physically keep the sample data with the actual sample. Location data, date of collection, name of the person collecting the sample, estimated scat age and any specific peculiarities of the sample were recorded at the time of collection. We also simplified returning of samples to the laboratory by providing pre-addressed, postage-paid envelopes in each kit that participants used to send the samples back through regular post. For this purpose we switched to using non-volatile, non-toxic buffer for sample storage (DEETs buffer) and did extensive testing of different sample collection tubes to get a robust, leak-proof vessel for safe mailing of samples through post. We selected a robust 8ml tube with a silicon seal which was also of convenient size for mailing. We also included two flat wooden sticks (ice cream sticks) with each tube to make sample collection easier. Out of the thousands of tubes that were received back in the laboratory through the post system, we haven’t had a single leak or complaint by the post service.

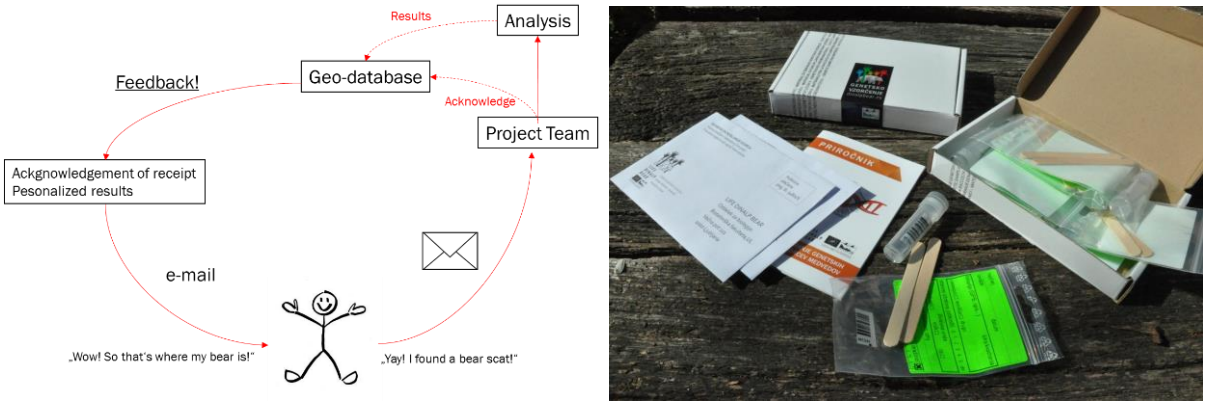

Figure 3: Individual level participation and feedback (left), personal sampling kit (right).

We tried to establish individual communication channels (through e-mail) with as many participants as possible, to establish individual-level participation. We used this for communication during the sampling to motivate participants, and we're using it once the analyses were finished to communicate the personal results for the samples that each participant collected (Figure 3). Each e-mail was tailored to the specific participant and provided a direct link (through the Monitoring Database developed in Action C.8) to information about the samples that he or she collected, the information about the samples collected by his/her hunting club, and information about the samples collected at regional and project level. We used special "bear sampling" T-shirts as a motivator for individual hunters to sign-up, and for promotion of the LIFE DINALP BEAR project; each person that took the sampling kit was invited to provide e-mail, address and t-shirt size to get access to personalized results and a t-shirt. All personal data is treated as confidential and archived in an appropriate manner – stored under password in cloud storage and accessed through encrypted connection.

Real-time monitoring of the sampling effort

We recorded all samples in the cloud-based geo-database immediately when they arrived. Having the database online enabled us to immediately record the samples at both locations where the samples were delivered (in each country) and share the data in real time. Geo-database automatically plotted the samples on maps, enabling us to constantly track the progress of sample collection. We regularly checked the maps to see if there are any "blank areas" where samples were not being collected to take appropriate action. In Slovenia we had to intervene in a couple of cases, but these were mostly mistakes and miscommunications, and sampling was kick-started with just a telephone call. In Croatia, there were areas where local hunters didn't collect samples at all. In these cases the project team members organized volunteers from broader public and/or sampled the area themselves. Keeping the track of sampling as it was progressing enabled us to address these issues before they would become a problem. In most cases we succeeded.

Collecting genetic samples of dead bears

Genetic samples of dead bears are routinely collected in both countries. Each detected bear mortality is sampled, weighted, measured and a tooth is taken for age determination. The latter is performed by the world-leading facility for age determination in wildlife from dentin layers in teeth, the Matson's laboratory in the USA.

Sample handling and storage, DNA extraction

While the study had the specific goal of estimating the population size of bears in Slovenia and Croatia, it also had the broader goal of providing foundations for efficient, reliable and cost-efficient long-term monitoring of this species in NW Dinaric Mts. and Eastern Alps. This meant that the laboratory methods needed to be updated to allow for cost-effective genotyping of large numbers of samples in short time. We needed to start implementing laboratory automatics to speed up the process and decrease labour.

Also, while microsatellites (short tandem repeat sequences) are currently the markers of choice for this type of studies, the usual manner of genotyping using capillary electrophoresis and fragment analysis is laboratory-specific, and results are not transferrable between laboratories unless stringent inter-laboratory calibrations are performed. There may even be issues of ensuring data consistency within a single laboratory if technicians or instruments change. After we started the project a new method of genotyping microsatellites using next-generation sequencing became available which solves all these issues by going to the most basic level – the level of the actual DNA sequence. It is also considerably faster, more reliable and less labor-intensive than the "classic" capillary electrophoresis. However, it is also new, and we were the first laboratory in the world to apply the method to a large real-world problem, which carried its own set of issues we needed to solve.

Sample handling and storage

When received in the laboratory, each noninvasive sample received a unique barcode to completely avoid manual labelling of samples and manual entry of sample ID. This barcode followed the sample throughout the analysis. The barcode was scanned and all the data about the sample entered into the online database (developed within C.8).

Noninvasive samples were collected in DETs storage buffer and stored in the same tubes they arrived in at -20°C until DNA extraction. After DNA extraction we kept the samples at -20°C until all downstream analyses were completed. All samples are stored in freezers dedicated to noninvasive and historic genetic samples either in the dedicated laboratory for low-quality DNA or in the areas where there is no possibility of contamination with PCR products. Extracted DNA is stored at -20°C following the same contamination prevention procedures.

Tissue samples of dead bears and blood samples collected at live captures of bears have a much higher quality and quantity of DNA than noninvasive samples and could be a possible source of contamination. For this reason they are handled in the "tissue laboratory", a separate facility that has lower contamination prevention requirements. Storage of the extracted DNA is also in this separate

facility.

We keep a genetic bank of all collected genetic material of brown bears. All DNA extracts are organized, entered into the database and tracked using barcodes. For tissue samples we also keep some original tissue whenever possible in case a re-extraction of DNA is required since DNA in tissue is much more stable over time than extracted DNA.

Laboratory organization and contamination prevention

DNA in noninvasive 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. 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 analyses. Noninvasive genetic samples are a difficult material that needs to be handled appropriately.

The “gold standard” for extracting DNA from scat samples is DNA Stool Mini Kit produced by Qiagen. However, this kit is time consuming to use (a skilled person can do DNA extraction from 23 samples + negative control in a working day) and relatively expensive. This makes DNA extraction a serious bottleneck in any medium or large scale study based on noninvasive genetic samples.

We purchased a liquid handling robot (Hamilton Starlet) using internal university funds, and DNA extraction modules using LIFE DINALP BEAR funds. We tested a number of magnetic-beads based DNA extraction kits to find the optimal protocol, using the Qiagen’s Stool kit as a reference. We also programmed and optimized DNA extraction protocols for the robot to achieve a robust, reliable and fast DNA extraction. 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”.

Genotyping

Upon serious consideration we decided to use a new method described by (De Barba et al., 2016) for genotyping. The method taps the power of next generation (high-throughput) sequencing (NGS), and promised to solve many problems that plagued the “standard” approaches (difficulty to compare results between laboratories, subjectivity in genotyping…), increase genotyping success, and

considerably speed up analyses while lowering the costs.

We started collaboration with the laboratory that was developing the method even before the method was published. The Laboratoire d'Ecologie Alpine (LECA) from Grenoble, France that developed the method is one of the leading world laboratories for conservation genetics and has been leading the technological development in noninvasive genetic sampling. We went there for study visits in 2015 and 2016 to get hands-on experience with the method and the required bioinformatics tools, and discuss the application of the method to a large-scale study.

The PCR conditions, primer sequences, tagging and pooling procedures are described in De Barba et al. (2017) and will not be repeated here – since the procedure is quite different than how genotyping is usually done, an interested reader is advised to study the referenced paper. In short, primer oligonucleotides are extended by DNA tags (short specific DNA sequences). Instead of two primers, a set of primers with different tags (24 F and 32 R in our case) is used for each locus. Each sample is amplified using primers with a unique combination of tags (the same at all analyzed loci) that will uniquely identify this specific sample in the sequence data obtained from a NGS run. In practice this means that each well in a PCR microplate will have a unique combination of primer tags. With this system we can uniquely label samples in eight 96-well microplates, or 768 samples. A critical step is preparation of tag-hybridization primer plates (microplates where in every well is a mix of primers for all loci in the multiplex and a unique combination of tags) since any pipetting errors at this stage can create considerable problems in downstream analyses. We solved this by using the liquid handling robot for primer plate preparation, which makes the probability of pipetting errors marginal.

We multiplex 13 microsatellite markers + sex id marker in a single PCR. PCR products of all samples from all eight microplates and with all markers are pooled into a single tube (library), purified with a Minelute Purification kit (Qiagen), quantified on a Qbit instrument and sequenced on an Illumina HiSeq sequencer, resulting in approximately 10 million DNA sequence reads per library. We put 12 or 13 libraries in a single HiSeq run.

Once the sequences are received (basically a large text file), bioinformatics tools are used to filter out sequences for individual samples and markers, and identify individual alleles. We used the bioinformatics tools developed by De Barba et al. (2016), but then programmed our own functions in R for allele calling. We also programmed functionality for management and visualization of these data into our laboratory database application (MisBase) that enabled us to visually check every genotype for accuracy.

We used a modified multi-tube approach (Adams & Waits, 2007; Taberlet et al., 1996) with up to 8 re-amplifications of each sample according to the sample's quality and matching with other samples. In the first screening we did 4 parallel repeated genotyping runs of each sample. A consensus genotype was produced, and quality index (Miquel et al., 2006) and maximum-likelihood reliability (Miller, Joyce, & Waits, 2002) were calculated for each sample.

We also use the NGS method for genotyping tissue samples, but we extend the panel of markers through use of "standard" capillary sequencer to 29 markers in total. The exact protocols for markers analyzed using the capillary sequencer are described in Skrbinšek et al. (2012).

Matching of samples with the same genotype and assigning individuals to samples

Although discovering samples that have the same genotype (and should in principle belong to the same individual) seems straightforward, this is not necessarily the case. Incorrect matching either "merges" the actual individuals if the information in analysed loci is too low, or creates "new" virtual individuals if the samples are erroneously considered to have different genotypes because of genotyping errors. The first problem decreases with increasing the number of loci used, however this exacerbates the second problem. Genotyping errors, even with the most strict quality assurance protocols, are unavoidable in noninvasive samples (Taberlet, Waits, & Luikart, 1999; Waits & Paetkau, 2005). Incorrect matching can cause considerable biases in mark-recapture estimates (Roon, Waits, & Kendall, 2005). A solution has been proposed to analyse the minimum number of loci that still provide enough resolution to reliable identify individual animals, minimizing the error (Paetkau, 2005). While this does make intuitive sense, the problem is that in noninvasive samples an odd locus will not amplify reliably in a sample, and even with low number of loci analysed the errors caused by allelic dropout remain a significant issue. In such case a large number of samples will get discarded, losing data, limiting the number of recaptures and decreasing the chances of a study's success, while much of the problem of incorrectly assigning individuals to samples will still remain. Also, some samples won't reach the genotype reliability criteria with any sensible amount of repeats, but may provide a reliable multi-locus genotype match with another, reliably genotyped sample. Another problem that we have not yet seen mentioned in the literature, but becomes very real when a large number of animals is included in the study, is the multiple-testing problem. Some measure of probability of identity between two animals (Waits, Luikart, & Taberlet, 2001) is typically considered to determine the number of loci required to obtain enough resolution to discern between animals, however such PID or PIDsib is valid only for a single comparison. In a study there are N*(N-1)/2 comparisons (where N is the number of individuals included in the study), so an appropriate multiple testing correction should be used to correct the PID and PIDsib values for the study. When N gets large, the resolution of a modest set of loci quickly becomes inadequate.

We took another approach of analysing a large number of loci and allowing for some mismatches resembling allelic dropout (a non-amplifying allele, which is the most common genotyping error in noninvasive samples - see (Broquet & Petit, 2004)). We used a large dataset of brown bears from the same population genotyped using tissue samples with a very low error rate (T Skrbinšek et al., 2012a) to explore distribution of mismatches, and used this mismatch distribution to set thresholds for allowable genotype mismatch. If the observed mismatches couldn't be caused by allelic dropout (e.g. 3 or 4 different alleles at the same locus in both samples) the samples were either considered to belong to different animals or additional evidence was collected through further repetitions of the genotyping procedure.

Mark-recapture analysis

We used several mark-recapture modelling approaches. We used the Capwire approach (C. Miller, Joyce, & Waits, 2005) with the R-package Capwire (Pennell, Stansbury, Waits, & Miller, 2013). We also used the generalized linear model approach with the information-theoretic model selection(Burnham & Anderson, 2002), as applied in program MARK (White & Burnham, 1999). As possibly the most robust model, we used the Chao's Mh model (Chao, 1987), which should also be robust to capture heterogeneity, and used the R package RCapture (Baillargeon & Rivest, 2007) to fit the models.

The Capwire models assume continuous sampling, which fits with how our data has been collected. An additional advantage of these models is that they are reasonably robust to capture heterogeneity. For Capwire, we used likelihood-ratio test to select between the even capture rate model (ECM) and the two innate rates model (TIRM). Capwire seems to be robust with considerable capture heterogeneity and in small populations (C. R. Miller, Joyce, & Waits, 2005).

MhChao model is robust across a broad range of conditions, and also has the advantage to allow for continuous sampling. Although it lacks precision and accuracy at low sampling intensity, its estimates improve considerably as the sample size increases. In small populations it is generally outperformed by other methods (particularly Capwire), but as populations get larger it is increasingly superior (C. R. Miller et al., 2005)

While the MARK approach requires discrete sampling sessions, this wasn't how we collected the samples in our study. However, we considered MARK for analysis of our data because of its well-developed model selection procedures and flexibility to include additional information about individuals, or groups of individuals, directly in the models. To fit this requirement, we considered the data collected within a certain time interval (sampling interval) as a single sampling session. This has the additional benefit that as the data gets aggregated into a smaller number of discrete sampling intervals, all captures of an individual animal within an interval will get aggregated into a single capture, lowering the capture heterogeneity and increasing robustness of the analysis. On the other hand, aggregation into sampling intervals invariably means loss of data (Petit & Valiere, 2006). To find the ideal limits of each sampling interval, we programmed a recursive optimization routine in R programming language (R Development Core Team 2016) which iterated through all possible combinations of uneven interval durations for a given number of intervals and found a solution with the minimal data loss and the maximum number of animals captured in each interval.

All the models we used assume a demographically closed population. Since sampling was relatively short and before reproduction and we believe that the majority of mortality was recorded and where possible either included into the dataset (as mortality on capture) or appropriately dealt with, we assumed that the sampled population should behave as demographically closed. Also, the entire sampled population is relatively well enclosed on three sides, with only a part of border with Bosnia and Herzegovina providing an option for closure violation. Since the population density of bears in that area seems low and the area affected by the edge effect is marginal compared to the entire sampled area, we believe that the effect of this closure violation is marginal.

The population is demographically completely open between Croatia and Slovenia, and the national border crosses bear habitat without providing any significant physical obstacles to bear movement. On the other hand, population size in each country is required for management purposes. Since this is at the core of the bear area and where some of the highest bear densities are, the issue of edge effect must be taken into consideration, especially since sampling intensity in Croatia was lower than in Slovenia. We ran models for each area, interchanging how samples of transboundary bears were included (either in Slovenia or Croatia). In both cases we estimated "superpopulation" for an area (population that also included transboundary animals), but because of the higher sampling intensity in Slovenia we can expect that more transboundary animals were captured in Slovenia, which would bias Slovenian estimates of the transboundary animals upwards, and Croatian estimates downwards. We used the correction proposed by (Wilson, Anderson, Journal, & Feb, 1985) to correct for the edge effect and estimate of the "moment" population size for each country (the number of bears expected to be present in a certain moment in our each country). We used detected pairwise distances between locations of samples of the same animal to calculate W, the width of the transboundary strip where the animals would have a non-negligible probability of being included in sampling on one or the other side. Because of expected differences in habitat use, W was calculated separately for each sex. To obtain the moment population size estimate, we used the A_s/A_t as the correction factor for our superpopulation estimate, where A_s is the surface area being sampled in one country, and A_t the total area including the edge strip in the other country.

Exploring basic population genetics parameters

While population genetics parameters of the Dinaric brown bear population are quite well explored (T Skrbinšek et al., 2012b), we re-checked them with a larger dataset available in this project. We also looked if there were any "outlying" genotypes that could indicate immigrants in the population and did a basic exploration of existence of population structure. We used R and package 'adegenet' (Jombart, 2008) for these analyses.

Estimating temporal variation in effective population size

To start understanding how management differences in Slovenia and Croatia effect the population at the most fundamental evolutionary level, we analysed the temporal variation in estimates of effective population size (*Ne*) – the paramount genetic index showing both the evolutionary potential of the population and its vulnerability to random genetic change and inbreeding. Effective population size can be without doubt considered the most important parameter for inferring a population's conservation status. We used one of the best explored methods for estimating *Ne*, the linkage disequilibrium based method (R S Waples, 2006). We organized samples in "yearly cohorts", which included all animals alive in that year (determined from the date of mortality and age of the animal) and aged between 0 and 3 years, meaning that they shouldn't be each other's parents, which should provide us with an estimate of *Nb*, the effective number of breeders (Tomaž Skrbinšek et al., 2012). Estimating the effective population size in species with overlapping generations (as the brown bear) is quite involved (Robin S. Waples, Antao, & Luikart, 2014). The LDNe approach used in our study to estimate *Ne* assumes discrete generations, which in the case of the brown bear is clearly violated (Tomaž Skrbinšek et al., 2012). However, (Robin S Waples & Do, 2010) discuss a reasonable conjecture that if the number of cohorts represented in a sample is approximately equal to the generation length, the LDNe estimate should roughly correspond to Ne in a generation. This conjecture was later by (Robinson & Moyer, 2013) through simulation testing and by (Robin S Waples, 2014) through examples from natural species. We take this into account in our yearly samples that include animals spanning approximately 1 generation (age 0 to 8), meaning that the absolute effective population size estimates of our study should be close to the actual *Ne*. On the other hand since all yearly samples include the same number of cohorts, they should be directly comparable, and provide a reliable depiction of how effective population size changes through time. We used R to prepare the data and analyse results, and program NeEstimator (Do et al., 2014) to estimate *Ne*.

Results and discussion

Improvement of laboratory procedures

Improvement of DNA extraction protocols

Since the workload in our Laboratory for Conservation Genetics at University of Ljubljana keeps increasing, we managed to obtain university funds to purchase a liquid handling robot. This is a universal, multi-purpose laboratory automation equipment that can speed-up and make more reliable several steps in laboratory analysis of genetic samples. It can handle large amounts of pipetting steps with minimal manual intervention. Since we use it with barcode-labelling and tracking of samples, it decreases the probability of pipetting errors (especially sample mix-ups as the most problematic ones) nearly to zero. While it is relatively demanding to program and optimize, it has shown to be extremely robust once the initial "breaking-in" is completed.

While we were planning to purchase a dedicated DNA extraction machine with LIFE DINALP BEAR funds, we found that such machines were either too low-throughput, difficult to use, locked to using proprietary reagents and protocols from a single manufacturer, or too expensive. On the other hand we were able to purchase for the same price all extension modules required to perform fully automated, fast DNA extraction with the pipetting robot that can be optimized to work with any magnetic-beads-based DNA extraction kit from any manufacturer. Additional advantage is also a measure of flexibility since the throughput of the machine can be doubled if the workload increases simply through purchase of additional pipetting channels.

Figure 4: Inside of the Hamilton Starlet liquid-handling robot in our laboratory during DNA extraction.

Since quality of the extracted DNA is the key parameter that all downstream analyses critically depend on, we invested considerable time and effort in selection and optimization of the methods. Currently

the method of choice for DNA extraction from scat samples collected in the field is the DNA Stool Mini Kit (Qiagen), which has proven to provide good results in many studies. However, since it is based on spin-column technology, it is difficult to automate on large scale, would be prohibitively expensive to automate on our liquid handling robot, and is most appropriate for manual use. It is also relatively expensive to buy.

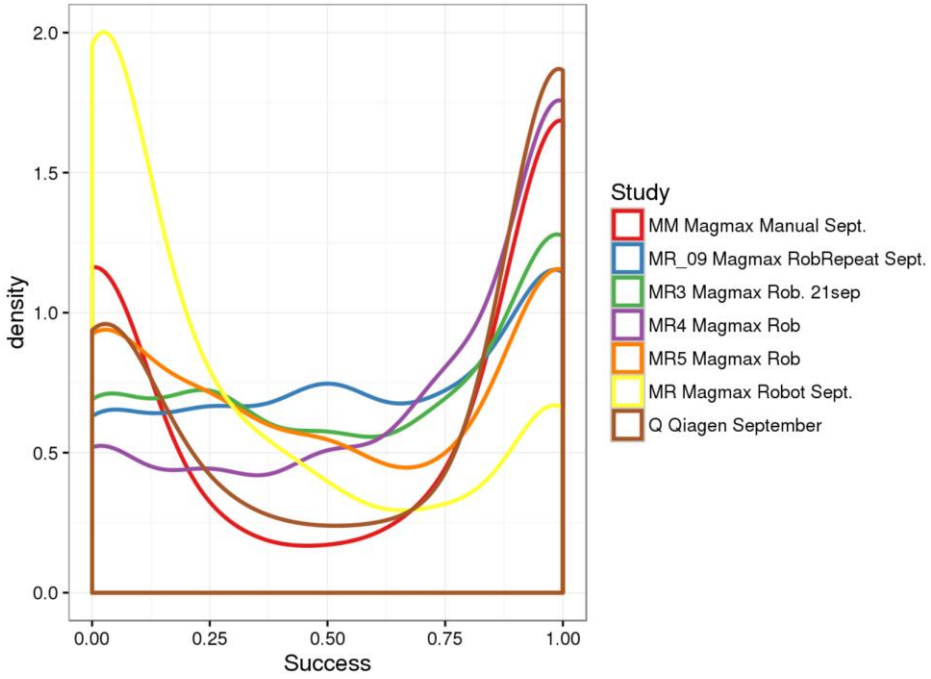

Figure 5: Tests of different automated protocols with MagMax DNA extraction kit (Applied Biosystem) and comparison with manual extraction and the 'gold standard' DNA Stool Mini Kit (Qiagen). Density plot, on x-axis is PCR amplification success using the extracted DNA. Same samples were used with each kit. The protocol MR4, which we later used for all DNA extractions, proved to have the same performance as the Qiagen's kit in high-quality samples (right side of the curve), but also outperformed the Qiagen's kit in low quality samples (a comparative shift of non-amplifying samples on left of the graph to the reasonably amplifying samples in the middle of the graph).

We tested 7 magnetic-beads-based DNA extraction kits, several with different extraction protocols, and compared performance using the same samples with the Qiagen's Stool Mini Kit. The best-performing kit, Mag Max (Applied Biosystems) we optimized to be used with the pipetting robot.

The final performance was actually somewhat better than with the Qiagen Stool Mini Kit (Figure 6). Also, while an experienced person can perform extraction of 23 samples + 1 negative control in a working day using the Qiagen DNA Stool Mini Kit, we can perform DNA extractions from 95 samples + 1 negative control per working day with the use of the liquid handling robot and magnetic beads extraction. There are also other advantages. Since all samples are tracked with barcodes and all pipetting steps logged, the probability of pipetting errors is next to zero. Actual DNA extraction is fully automated, meaning that expert laboratory staff is free to do other things. The manual work of preparing scat samples for extraction remains, but is very simple and can be done by non-expert staff (in our case mostly students paid through student assistance). This increases reliability and quality of work, and decreases costs.

Laboratory capacity gains and foundation for long-term genetic monitoring

With use of laboratory robotics and the switch to next-generation sequencing (NGS) for genotyping, we considerably decreased the workload of manual work in DNA extraction and allele calling (which was mostly done manually before, but is now handled by bioinformatics). This resulted in much shorter analysis turnover time and a decrease in costs. While getting the new technologies to work involved a considerable investment of time and effort and initially caused a large delay in processing of samples, the gains in analysis speed helped us recover the lost time and finish the study with a relatively small delay. The decrease in costs as well as smaller workload on the personnel ultimately made possible analysis of a considerably larger number of samples than what we originally planned in the project.

Table 2: Performance comparison of the 'standard' genotyping methods we used before upgrading, and the 'new' methods used to analyze samples in this study. To our knowledge we are the first laboratory to apply NGS genotyping of microsatellites in a large-scale study. Throughput estimates are all based on a single person performance, with some non-expert help (in our laboratory usually students) in sample preparation for DNA extraction.

Item	Previous method	"New" method
DNA extraction	Manual, spin-column based extraction, **23 samples/day + 1 NC**	Automated DNA extraction with a liquid handling robot, **95 samples/day + 1 NC**.
Genotyping	Capillary electrophoresis & computer-assisted allele calling, **30-40 samples/day**.	Next generation sequencing and automatic allele calling from sequences, **300-400 samples/day**.* *Can still be increased several fold with a relatively modest investment.
Transferability of data	Subjective, laboratory-specific allele calling, **poor transferability** of the data.	Objective, sequence-based allele calling, **complete transferability** of the data.
Future-proofing for longitudinal studies	Data problematic for longitudinal and time series studies even within a single laboratory (changes in equipment and personnel can introduce inconsistencies in the data).	Data at the most basic level, the DNA sequence. Completely future proof, just as useful 100 years from now as it is in this moment.

Sample collection and inclusion of volunteers

The action started on 5 Sep 2016, and lasted until 31 Dec 2016. We distributed close to 18,000 sampling tubes to the field to cover the entire study area. We never expected to get more than 20% back, but this "oversaturation" was required to equalize the sampling effort regardless of the variance in bear population density, which is an important factor for getting data suitable for mark-recapture modelling.

We recorded all samples in the geo-database immediately when they arrived. Geo-database automatically plotted the samples on maps, enabling us to track the progress of sample collection in almost real-time. We regularly checked the maps to see if there are any "blank areas" where samples are not being collected to take appropriate action. In Slovenia we had to intervene in a couple of cases, but these were mostly mistakes and miscommunications and sampling was kick-started with just a telephone call. In Croatia, there were areas where local hunters didn't collect samples at all. In these cases we organized volunteers from broader public and/or sampled the area using project personnel. Keeping the track of sampling as it was progressing enabled us to address these issues before they would become a problem and influence the final result.

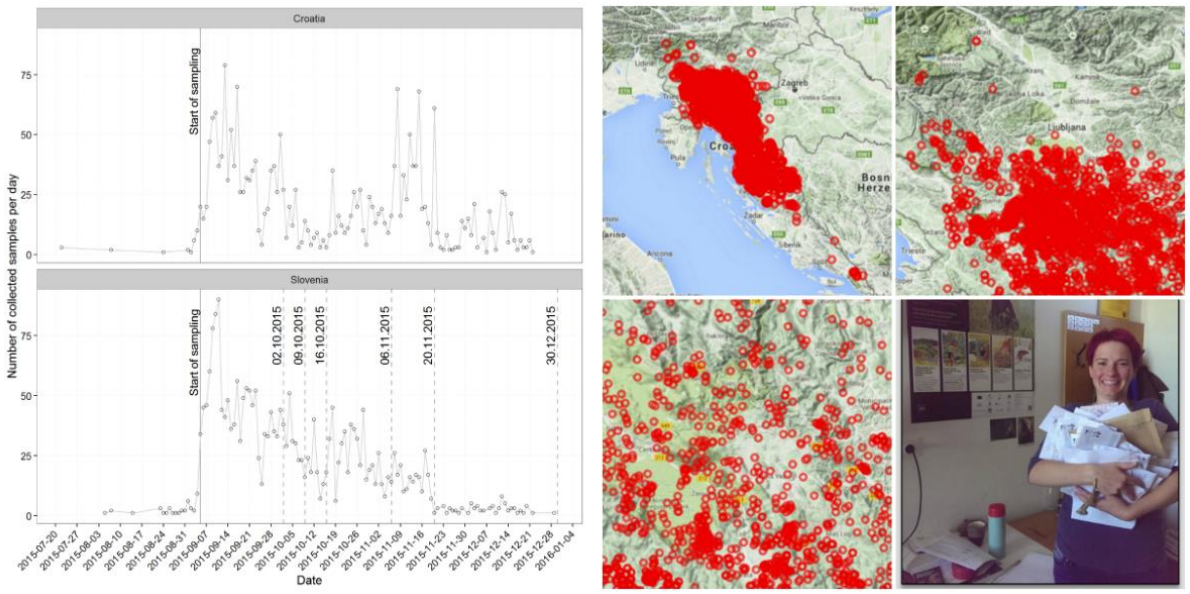

Figure 6: Number of samples collected per day in each country (left); spatial distribution of collected sampled (direct view in »mbase.org« geo database) and our team member Maja Jelenčič with a daily »harvest« of Slovenian samples from our mailbox (Croatian samples were being delivered to Zagreb).

Altogether, we managed to collect 4687 samples, with good temporal and spatial coverage (Figures 6 and 8). This is 56% more than the target 3000 we had planned in the project. Most samples are estimated as very fresh and we can without doubt call the action a complete success. The majority of samples were collected before the end of November when the first snows started. We had 962 participants that returned samples to us, and we estimate that over 2500 people were actively participating in the study (not all of them were able to find samples, also because many are in the areas

where bears occur sporadically). Interestingly, while the majority of participants in Slovenia (estimated 87.5 %) actually registered in the geo-database, this proportion is much lower in Croatia (estimated 17 %). Slovenian hunters have already participated in a similar study in 2007, they received a feedback and recognition for their efforts and there is a long-standing trust between researchers and people in the field. We feel that in Croatia this trust still has to be built, and we hope that this sampling action is providing an important first step in this direction.

Table 3: The numbers of participants that actually provided samples (per country) and the numbers of participants that registered in the geo-database. We used a simple Lincoln-Petersen mark-recapture model to estimate the total number of participants participating in the study.

Number of participants that provided samples			
Country	Registered in the database	Not registered in the database	Total
Croatia	70	341	411
Slovenia	482	69	551
Total	552	410	962

		Registered and estimated total number of participants included in the study	
Country	Total registered in the database	Proportion of registered participants that provided samples	Estimated total number of participants
Croatia	225	17.0%	1321
Slovenia	1098	87.5%	1255
Total	1323		2576

Genotyping

We have two major sample sets for genotyping, with small overlap. One are the noninvasive samples collected to estimate the number of brown bears in Slovenia and Croatia. The other are tissue samples collected over long time that we're using to monitor temporal and spatial variation of effective population size. The small overlap are samples of the bears that died during the intensive sampling in late 2015.

Genotyping of noninvasive genetic samples

Although we originally planned to process 3000 samples of the intensive genetic sampling in Slovenia and Croatia, the advances in analytical procedures allowed us to process 4370 samples, or 45.7% more than planned. This alone shows the importance of the new methods for large-scale genetic monitoring of wildlife, and justifies the effort dedicated to their implementation. All in all we managed to genotype 93% of all collected samples. The remaining samples are relatively old (lower success rate), and are all in Slovenia where sampling was much more intensive and additional samples would have negligible effect on results.

We managed to successfully genotype 3218 samples, or 73.6% of processed samples. 34 of these samples were not used in the downstream analyses since the genotype reliability was not high enough, but further genotyping was not done because of time considerations and because the number was low enough for the effect on the entire study to be negligible.

Parallel to noninvasive samples we also genotyped samples of all detected bear mortality, 142 individuals. This yielded the total number of 3326 samples for downstream analyses.

Genotyping of tissue samples of detected bear mortality

Tissue samples are routinely collected both in Slovenia and Croatia for over a decade. They have also been genotyped, but not all and not systematically, which we're fixing in this project.

The total bear mortality dataset currently includes genotyped samples of **2022 individual bears** (1040 from Slovenia and 982 from Croatia). We've been routinely using a 16-locus panel + a sex-id marker for genotyping using capillary electrophoresis. We re-analysed all samples collected prior to this project (1326 samples) with the new NGS markers, both to ensure compatibility with the noninvasive dataset and to increase the information content for downstream analyses. This brings the total dataset to **29 polymorphic microsatellite loci** + 2 loci for confirmation of field determined sex id.

In LIFE DINALP BEAR we collected and fully analysed 696 of tissue samples (331 from Slovenia and 365 from Croatia) on all 29 loci.

There are still 446 samples in the process of being genotyped at the time of writing of this report. Since bear mortality continues to be sampled, we will analyse new tissue samples throughout the project for other purposes. However, this is already one of the largest brown bear datasets in the world, and more than adequate fort the purpose of estimating temporal variation in effective population size.

Movement of animals for estimation of transboundary animals

We used pairwise distances between samples of the same animal to understand movement of animals, and determine the number of transboundary animals. There is a considerable difference between movement of males and of females, so corrections were done separately for each sex (Figure 7).

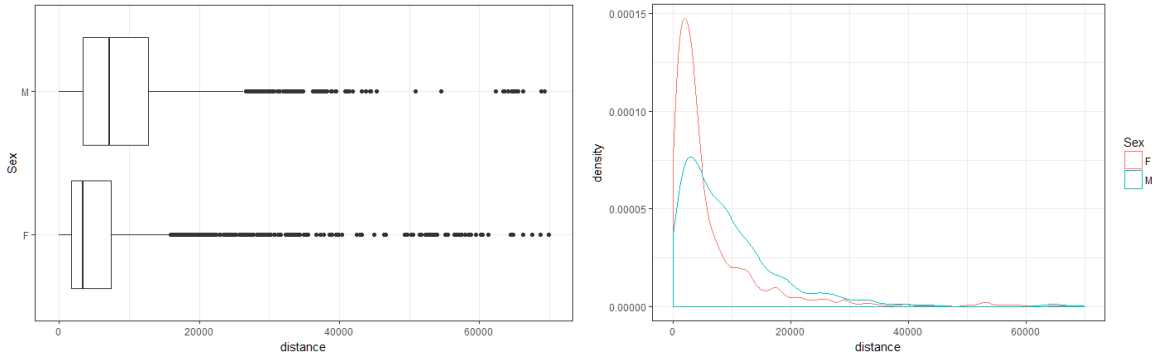

Figure 7: Pairwise distances between samples of the same animal, by sex.

Population size estimates and sex ratio for the entire study area

Successfully genotyped samples, captured animals and mortality

The sampling results for the entire area are summarized in Table 3 below. While the total number of successfully genotyped samples was 3326, we removed 30 samples (0.9 %) where geographic location was not recorded from the downstream analyses. We also removed 33 samples (0.9 %) that didn't match any other samples and had quality index below 0.8. The number of removed samples is marginal, and the resulting dataset with 3263 samples should be error free.

Table 4: Genotyped samples, numbers of captured animals and mortality for the entire study area.

Samples and captured animals	
All genotyped samples	3263
Total captured animals	1136
Males	467
Females	669
Total recaptured animals	730 (64.3 %)
Mortality	
Total mortality during sampling (Sep 2015 – Dec 2015)	142
Males	79
Females	63
Total mortality in the study area in 2015	256

Spatial distribution of samples was good (Figure 8), with the exception of Eastern Lika area in Croatia where intensity of sampling was lower, but still acceptable.

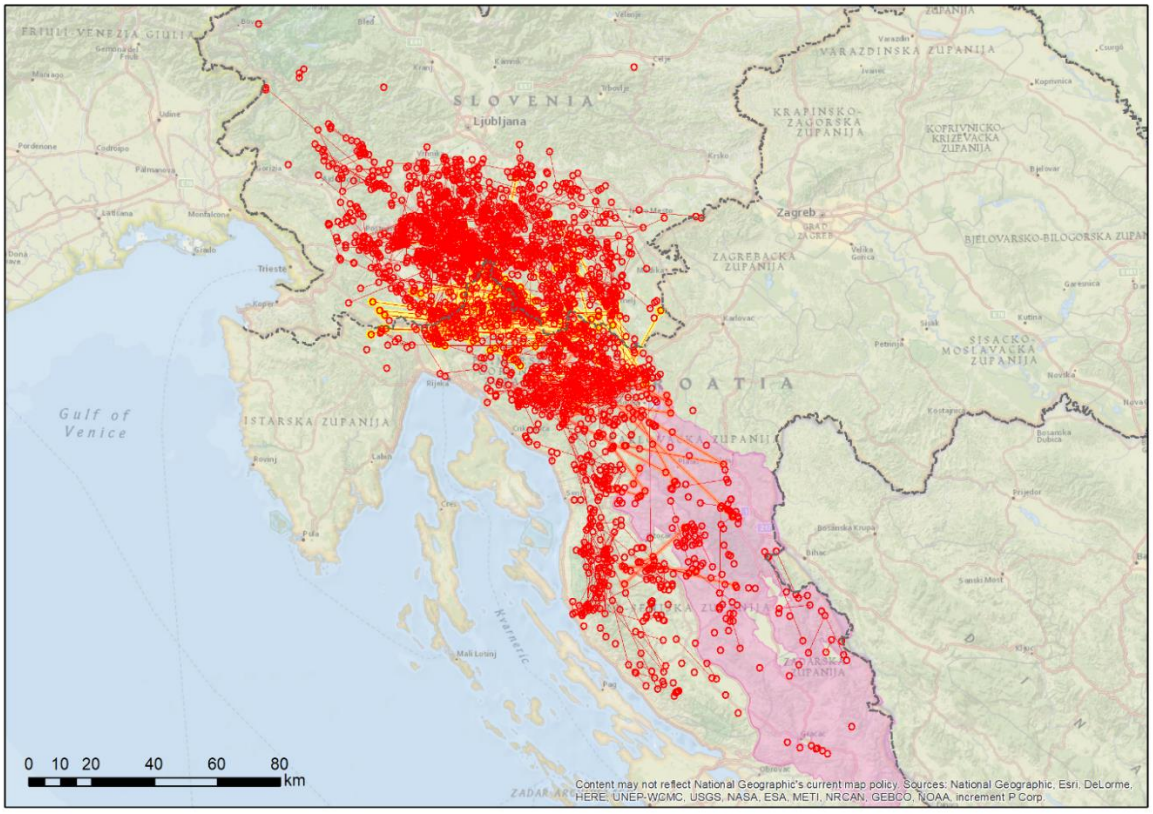

Figure 8: Successfully genotyped samples. Lines connect samples of the same animal (paths), paths of transboundary animals between Slovenia and Croatia are marked yellow. The area of Eastern Lika where sampling intensity was lower is marked in pale red, and paths of animals crossing in and out of that area outlined orange.

Temporal distribution of samples was also good (Figure 9), with the majority of samples collected before the end of November, and just a couple of samples collected afterwards.

Mark-recapture estimates of population abundance for the entire study area

At the level of the entire study we expected to see capture heterogeneity since both the number of samples as well as recapture rate varied among different areas. We took four different approaches to mark-recapture modelling:

1. All samples were considered together in the same model.
2. Each sex was modelled in a different model (hypothesis: capture rate varies between sex).
3. Each area that seemed to have a different sampling intensity was modelled in a separate model (hypothesis: capture rate varies between areas).
4. Different models were made for each area and each sex (hypothesis: capture rate varies by area and by sex).

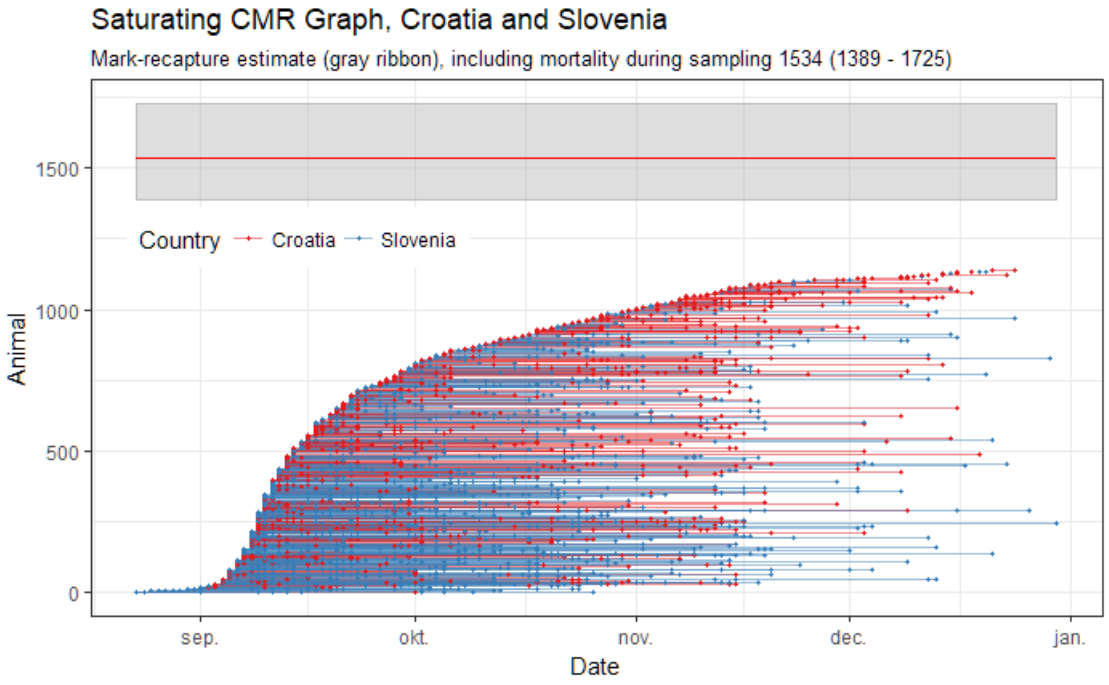

Figure 9: Saturating Mark-Recapture Graph for the entire study area - samples and captured animals ordered by the date of first capture. Each dot is a sample, each line connects samples of the same animal, and time goes left to right. We can see saturation – less and less new animals appear in new samples. The estimated number of animals that were never captured is the difference between the estimated total population size (red line and gray ribbon of confidence interval at the top) and the top of the mark-recapture graph. The graph includes 142 recorded mortality during sampling.

We used several modelling approaches to estimate abundance of brown bears in the entire study area.

With continuous-sampling models (Capwire, MhChao), there are technical limitations to including all these hypotheses in a single model set and compare support in the data with any statistically stringent model selection approach. We were able to do this in MARK, but these models are capture-session based and it can be expected that they don't use the continuous sampling data efficiently (Petit &

Valiere, 2006). Although the large sample size if all the samples are used in a single model provides a narrower confidence intervals, we preferred the "composite" estimates with different models for areas with different sampling intensity since the assumption of capture homogeneity is in this manner the least violated.

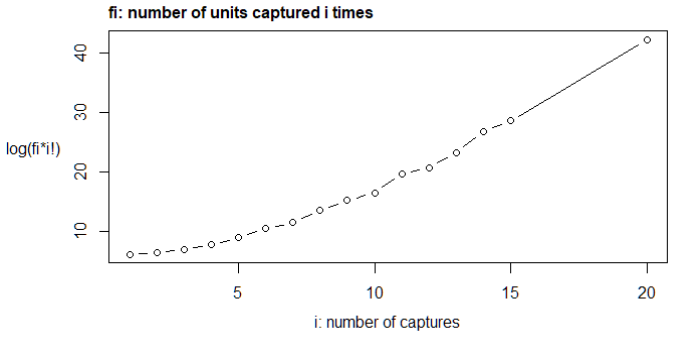

Figure 10: Exploratory heterogeneity graph for the entire study area (produced using Rcapture). Under capture homogeneity assumption this graph should be linear. While there is some capture heterogeneity in the data, it seems to be relatively low.

We considered the models that assumed no capture heterogeneity (M0, Capwire ECM), but they had no support in the data and will not be shown here. An exception here is the Huggins model which doesn't assume heterogeneity, but we were able to explicitly model capture probability by sex, time and area. We also tested the Huggins heterogeneity model (which models heterogeneity as two finite mixtures) which provided a significantly lower AIC, but had estimability problems (unreasonably high or non-estimated standard errors). The Huggins model with sex.area.time parametrization provided results that were very similar to other models we used for the final estimate and we believe that we were able to include most of capture heterogeneity in the model parameterization.

While models that considered hypothesis 2 (capture probability varies by sex) were selected through model selection in MARK (Huggins model), the estimates by each sex added to nearly exactly the same value as the estimates for both sexes together. We used the sex-specific estimates to estimate sex ratio, but are not showing here the sex specific results.

Since capture probability varied considerably among areas, as evident from different recapture rates, it made sense to explicitly model different areas (hypothesis 3). We did this through several approaches, and the one we thrust the most is using the MhChao model for Slovenia and West Croatia, and Capwire TIRM for Eastern Lika. MhChao has superior performance when population and sample size is large, which is the case in most of our study. It also has conservative (large) confidence intervals. Since we were curious about the effect of sample size on performance of different models, we did multiple resampling of the dataset to smaller sample sizes (down to 50% of all samples, not shown here). In all models the estimates increased with the sample size, but MhChao proved to be the most robust, and provided reliable results and good confidence interval coverage even at the lowest resampling. This made it the model of choice for most areas in our study.

Since both sampling intensity and the number of samples were low in Eastern Lika, we modelled that area separately using Capwire TIRM model, which in such situations outperforms the MhChao model (C. Miller et al., 2005).

Another consideration is edge effect and its correction. Since the population is open towards Bosnia and Herzegovina, the estimates are a "superpopulation" estimate meaning that they also include animals that have a non-negligible proportion of their homerange outside of the study area. There are methods how to take this into consideration, as we did to provide country-specific estimates for Slovenia and Croatia.

In the case of border with Bosnia and Herzegovina, we decided not to correct for edge effect for several reasons:

- The "edge" area is relatively small compared to the entire study area.
- Bear abundance in the border area in Eastern Lika is relatively low.
- Sampling in the border area in Eastern Lika has been of lower intensity, which means that transboundary animals (which were not present in the study area entire time) were probably underrepresented in the sample.
- The exact situation of brown bears on the other side of the border is largely unknown to us.

While we understand that not correcting for edge effect may cause a slight population overestimate, this is well within the statistical error of our estimate. Also, if we consider the estimated number of transboundary animals between Eastern Lika and the other bear areas in Croatia, which we estimate to be 65 animals (61-84 95% CI) and the geography of the area, this overestimate should not be more than 30 animals, but is quite likely much less.

Since there is high anthropogenic mortality in the study area, even assuming a stable population the number of brown bears varies considerably during the year. We estimated two abundances – the maximum and minimum yearly abundance.

The minimum yearly abundance was estimated directly through mark-recapture, which was done at the end of the year. The mortality detected during the sampling was subtracted from the mark-recapture estimate to obtain the final value. This is the end-of-the-year estimate for 2015, after mortality and before next reproduction.

The maximum yearly abundance is derived from the minimum yearly abundance through addition of all detected mortality in that year. In our case it means the estimated abundance of brown bears in spring 2015. It is an underestimate since it doesn't take into account undetected mortality, which is assumed to be relatively low in brown bears (with the possible exception of the cubs of the year which may go undetected).

Table 5: Mark-recapture estimates of abundance of brown bears in 2015 in NW Dinaric Mountains and Eastern Alps of Slovenia and Croatia. The model that we believe to be the best is in bold print. The minimum yearly estimate is the end-of-the-year abundance for 2015, after mortality and just before reproduction (in early 2016). It is the direct mark-recapture estimate minus the animals that died during sampling. The maximum yearly abundance is abundance in spring 2015, and is the minimum abundance plus all recorded mortality in that year. The maximum abundance is underestimated since it doesn't include undetected mortality, but this is assumed to be relatively low in brown bears.

CMR Model	Minimum Yearly N (95% CI)	Maximum Yearly N (95% CI)
Capwire TIRM	1442 (1424-1529)	1698 (1680-1785)
Huggins	1434 (1370-1497)	1690 (1626-1753)
MhChao	1312 (1233-1391)	1568 (1489-1647)
MhChao+Capwire TIRM	**1392 (1247-1583)**	**1648 (1503-1839)**

Sex ratio for the entire area

We took two approaches to estimating sex ratio: directly from the sex ratio of captured animals, and from mark-recapture estimates for each sex. Both methods provide very similar results. Direct estimate is intuitive, uses empirical data directly, is simple and easy to understand, and there is no indication of variance in capture probability by sex, hence we used it as the final sex ratio estimate.

Table 6: Sex ratio for the entire study area, model and direct estimates.

Area	CMR Model	Female	Male
All	Capwire TIRM	0.583	0.417
All	MhChao	0.572	0.428
All	**Direct Samples**	**0.589**	**0.411**

There are considerably more females (59%) than males (41%). Considering higher mortality among males this is an expected result.

Population size estimates and sex ratio for Slovenia

Sampling in Slovenia was incredibly intensive, and the number of samples collected exceeded expectations. This is reflected also in the high recapture rate. Results are summarized in Table 6.

Table 7: Genotyped samples, numbers of captured animals and mortality for Slovenia. The total number of samples includes also samples of transboundary animals in Croatia.

Samples and captured animals	
All genotyped samples (including transboundary)	1962
Total captured animals	614
Females	366
Males	248
Total recaptured animals	427 (69.5 %)
Transboundary recaptured animals	14.1 %
Mortality	
Total mortality during sampling (Sep 2015 – Dec 2015)	65
Females	27
Males	38
Transboundary, mortality in HR	+5 (2F, 3M)
Total mortality in Slovenia in 2015	112 (67M, 45F)

Spatial distribution of samples was good and covered the entire brown bear range in the country (Figure 11).

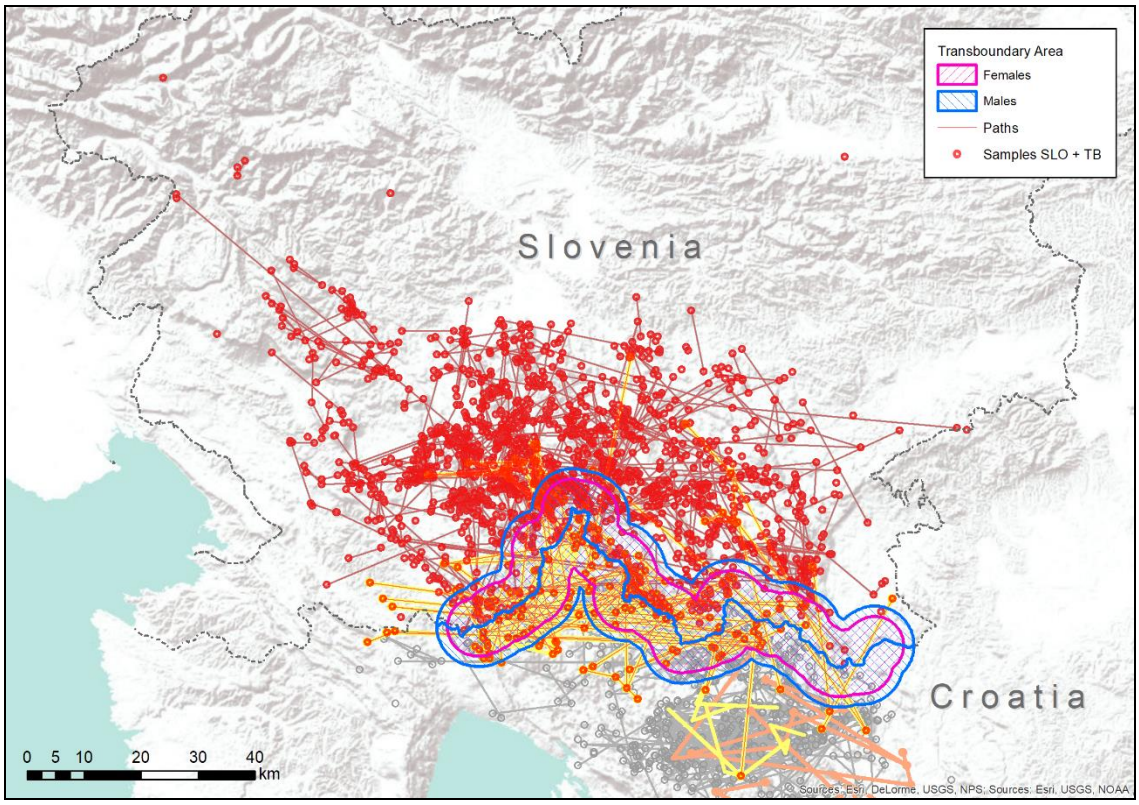

Figure 11: Spatial distribution of samples in Slovenia. Lines (paths) connect samples of the same animal, paths of the detected transboundary animals are highlighted yellow. The transboundary area was determined using pairwise distances between samples of the same animal.

Since recapture was very high, the saturation graph (Figure 12) seems to be approaching saturation, with a low number of individuals estimated never to have been captured. Capture heterogeneity also seems low (Figure 13).

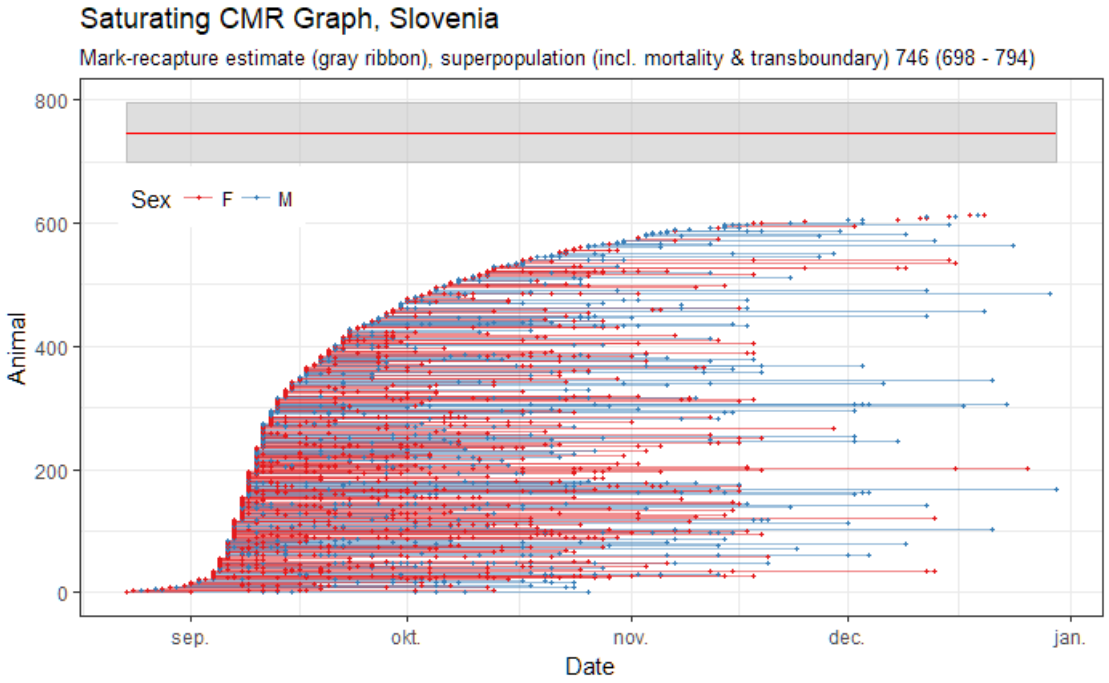

Figure 12: Saturating Mark-Recapture Graph for Slovenia - samples and captured animals ordered by the date of first capture. Each dot is a sample, each line connects samples of the same animal, and time goes left to right. We can see saturation – less and less new animals appear in new samples. The estimated number of animals that were never captured is the difference between the estimated total population size (red line and gray ribbon of confidence interval at the top) and the top of the mark-recapture graph. The graph includes 70 recorded mortality (also 5 transboundary animals that died in Croatia) during sampling.

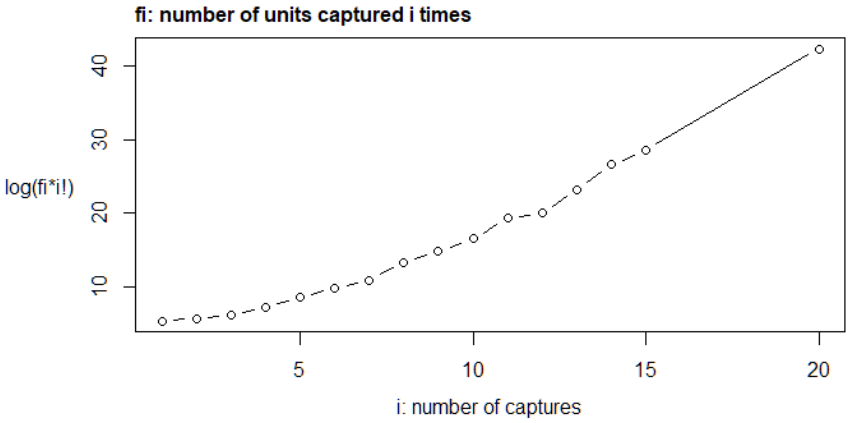

Figure 13: Exploratory heterogeneity graph for Slovenia + transboundary samples (produced using Rcapture). Under capture homogeneity assumption this graph should be linear, and in this case capture heterogeneity seems low.

We estimate that there are 135 (121 – 147) transboundary animals through movement data, and we get similar results through proportion of transboundary animals in in all recaptured animals in the core areas close to the border (an animal must be recaptured to be detected as transboundary). Since sampling intensity is higher in Slovenia, the expectation is that the higher proportion of transboundary animals is captured in Slovenia. Looking at differences in superpopulation estimates (abundance that includes transboundary animals) for Slovenia and Western Croatia versus population estimates for pooled samples from both areas the proportion of transboundary animals in superpopulation estimates for Slovenia is 56.9% (77 individuals), and we used this to correct the superpopulation estimate for Slovenia and get the moment estimate of the number of bears that could be expected in Slovenia in a certain moment in time.

Table 8: Mark-recapture estimates of abundance of brown bears in 2015 in Slovenia. The model that we believe to be the best is in bold print. The minimum yearly estimate is the end-of-the-year abundance for 2015, after mortality and just before reproduction (in early 2016). It is the direct mark-recapture estimate minus the animals that died during sampling. The maximum yearly abundance is abundance in spring 2015, and is the minimum abundance plus all recorded mortality in that year. The maximum abundance is underestimated since it doesn't include undetected mortality, but this is assumed to be relatively low in brown bears.

Statistical Model	Minimum Yearly N (95% CI)	Maximum Yearly N (95% CI)
Capwire TIRM	622 (611-688)	734 (723-800)
Huggins	589 (566-615)	701 (678-727)
MhChao	**599 (545-655)**	**711 (657-767)**

Sex ratio estimated only for Slovenia does not differ significantly from sex ratio estimated for the entire study area (Table 8). Since males have larger homeranges, their proportion may be overestimated. This is expected to be worse for model estimates since mark-recapture models for males will be more effected by the edge effect than the models for females, making the direct assessment from samples (in bold) more reliable.

Table 9: Model-derived and directly estimated sex ratio for Slovenia. Since males have larger homeranges, their proportion may be overestimated. This is expected to be worse for model estimates since mark-recapture models for males will be more effected by the edge effect than the models for females, making the direct assessment from samples (in bold) more reliable.

Area	Model	Female	Male
Slovenia	Capwire TIRM	0.589	0.411
Slovenia	Huggins	0.583	0.417
Slovenia	MhChao	0.568	0.432
Slovenia	**Direct Samples**	**0.596**	**0.404**

Population dynamics of brown bear population in Slovenia

While the estimate for the entire NW Dinarics area has not been done before, a genetic estimate of the brown bear population size in Slovenia has been performed in 2007 using very similar methods to the ones we're using in this study (Tomaž Skrbinšek et al., 2008). This allows us to add temporal component and move from a population "snapshot" towards actual monitoring of the population.

The population size for Slovenia, minimum yearly population size corrected for edge effect of transboundary animals shared with Croatia, was in 2007 estimated 424 (383-458) bears. The methodologically very similar estimate obtained for 2015 in this study is 599 (545-655) bears, or a 41.3 % increase over the period of 8 years.

Since less samples were collected in the 2007 study, we tested sensitivity of the models to sample size by randomly resampling Slovenian genotypes without replacement to smaller sample sizes (from 50% to 95 % of all samples in 5% steps, 100 re-samples in each step) and re-estimating population size form each such subsample. While smaller sample sizes did cause a downward bias in the estimates, this effect was marginal and we were able to get a robust result (but with a larger confidence interval) even with as little as 50% of actually genotyped samples.

Population size estimates and sex ratio for Croatia

Sampling in Croatia was less intensive than in Slovenia, but the number of collected samples was still very large. However, while sampling coverage was good in most areas, it was less intensive in Eastern Lika area (Figure 14). This meant that capture probability of animals in Eastern Lika was lower, driving capture heterogeneity at the level of the entire country that we solved by explicitly modelling Eastern Lika as a separate entity or including the area information in the models (in the MARK models that allow including such complexity).

Table 10: Genotyped samples, numbers of captured animals and mortality for Croatia. The total number of samples includes also samples of transboundary animals in Slovenia.

Samples and captured animals	
All genotyped samples (including transboundary)	1539
Total captured animals	582
Females	339
Males	243
Total recaptured animals	361 (62 %)
Transboundary recaptured animals	16.6 %
Mortality	
Total mortality during sampling (Sep 2015 – Dec 2015)	77
Females	36
Males	41
Transboundary, mortality in SLO	2 (1F, 1M)
Total mortality in Croatia in 2015	144 (92M, 50F, 2 unknown sex)

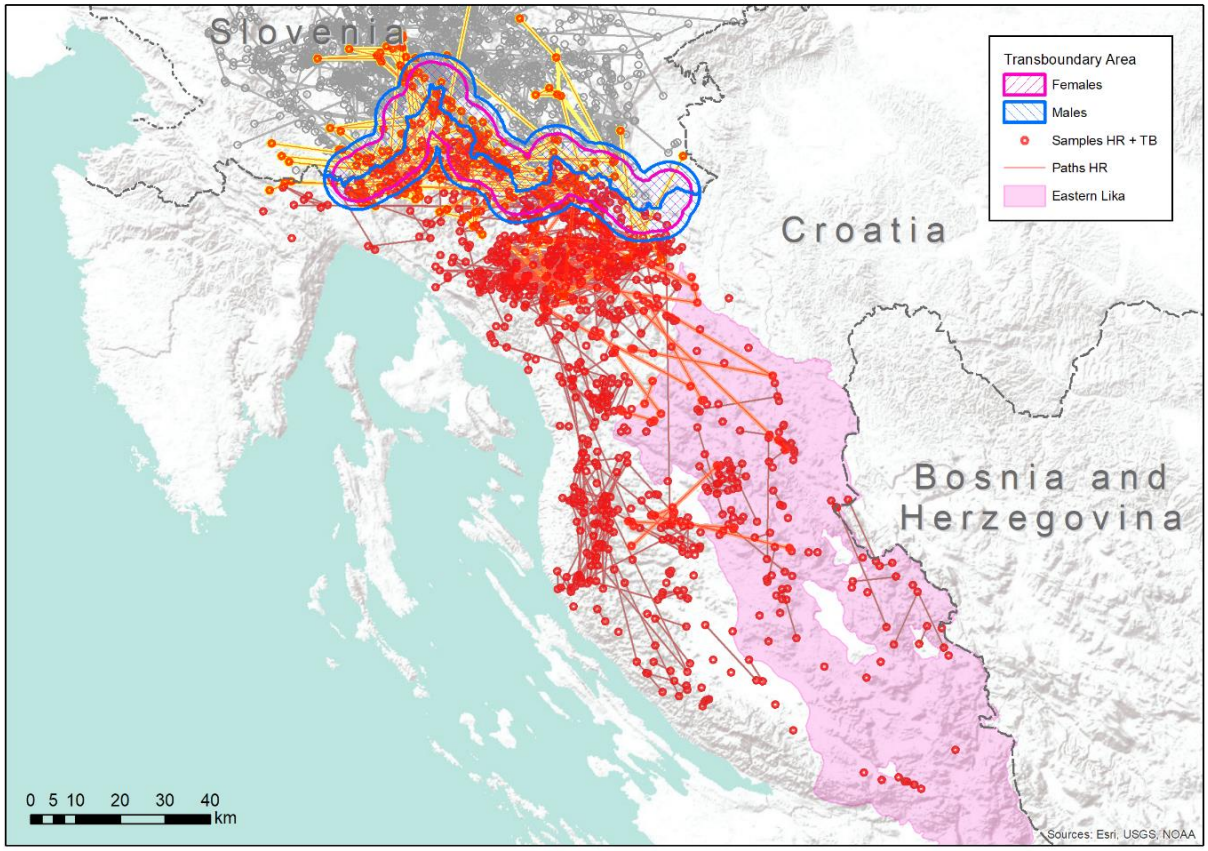

Figure 14: Spatial distribution of samples in Croatia. Lines (paths) connect samples of the same animal, paths of the detected transboundary animals are highlighted yellow. The transboundary area was determined using pairwise distances between samples of the same animal. Eastern Lika, where sampling intensity was lower, is shown in light red.

Although sampling was reasonably successful over most of the study area in Croatia, there were areas where at the beginning it wasn't going well. This was detected through real-time monitoring of sample collection, and steps were taken by the project team to improve coverage in such undersampled areas as much as possible. We can see this in the saturating CMR graph for Croatia (Figure 15) as a "notch" in mid-October when action was taken to improve sampling coverage.

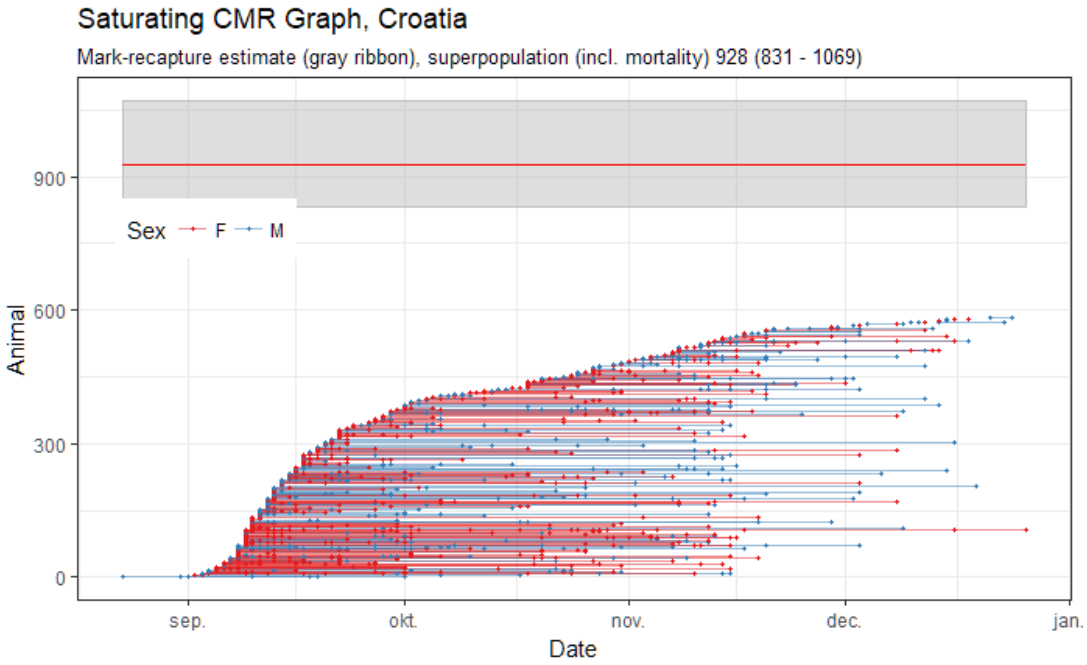

Figure 15: Saturating Mark-Recapture Graph for Croatia - samples and captured animals ordered by the date of first capture. Each dot is a sample, each line connects samples of the same animal, and time goes left to right. While we can see saturation – less and less new animals appearing in new samples – it is less evident than in Slovenia. We can also see a "notch" in mid-October when action was taken to cover undersampled areas. The estimated number of animals that were never captured is the difference between the estimated total population size (red line and grey ribbon of confidence interval at the top) and the top of the mark-recapture graph. The graph includes 77 recorded mortality (also 2 transboundary animals that died in Slovenia) during sampling.

Looking only at the areas besides Eastern Lika (SW Croatia) we can see that capture intensity is relatively high, and the proportion of recaptured animals very close to that achieved in Slovenia (68.7 % vs. 69.6 %, Tables 6 and 10). On the other hand only 47.5 % of animals in Eastern Lika were recaptured, indicating lower sampling intensity.

Table 11: Genotyped samples, numbers of captured animals and mortality for different areas in Croatia. SW Croatia means all bear range except for Eastern Lika (see Figure 14 for map). For SW Croatia, the total number of samples includes also samples of transboundary animals with Slovenia.

Samples and captured animals – SW Croatia	
All genotyped samples (including transboundary)	1373
Total captured animals	496
Females	289
Males	207
Total recaptured animals	341 (68.7 %)
Transboundary recaptured animals (with SLO)	17.6 %
Samples and captured animals – Eastern Lika	
All genotyped samples (including transboundary)	203
Total captured animals	99
Females	57
Males	42
Total recaptured animals	47 (47.5 %)
Transboundary recaptured animals (with SW Croatia)	27.7 %

This lower intensity is also evident from saturation graphs (figure 16). There was considerable capture heterogeneity in Eastern Lika (Figure 17), probably since when looking at spatial distribution of samples (Figure 14) some parts of this area seem to have been sampled more intensively.

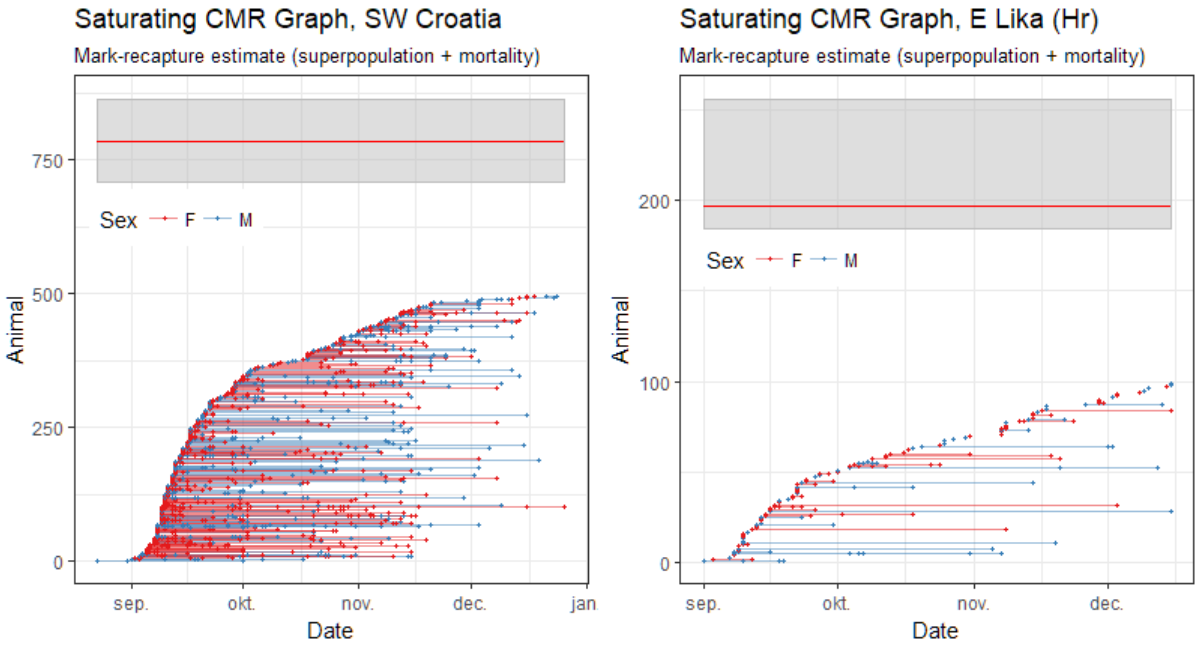

Figure 16: Comparison of Saturating Mark-Recapture Graphs for SW Croatia and Eastern Lika. While we can see saturation in the rest of the bear area in Croatia, recapture in Eastern Lika is considerably lower, and the difference in the total number of captured animals and the estimated abundance much higher.

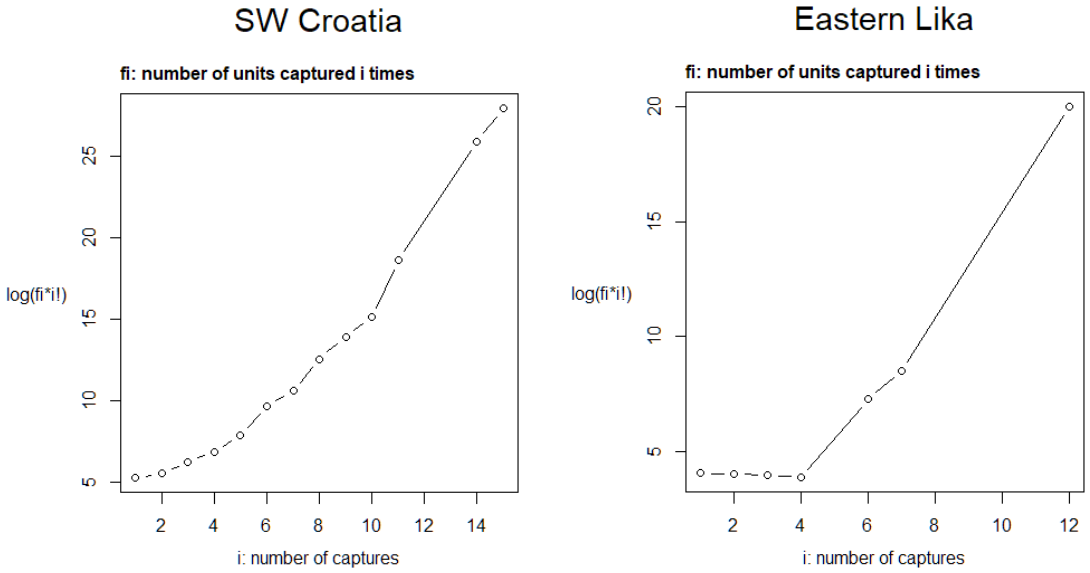

Figure 17: Exploratory heterogeneity graphs for SW Croatia and Eastern Lika. With no capture heterogeneity this plot should be linear. Plot for SW Croatia indicates moderate capture heterogeneity, while the situation in Eastern Lika seems quite severe.

The only reasonable mark-recapture modelling approach was to model both areas separately. While all candidate models provided similar results for the well-sampled areas, we used Capwire TIRM for Eastern Lika since it should perform best in small populations with high capture heterogeneity. By-area results are summarized in Table 11.

Table 12: Mark-recapture estimates of abundance of brown bears in 2015 in SW Croatia and Eastern Lika. The model that we believe to be the best is in bold print.

Statistical Model	Minimum Yearly N (95% CI)	Maximum Yearly N (95% CI)
SW Croatia		
MhChao	**646 (577-719)**	**707 (638-780)**
Huggins	651 (606-700)	712 (667-761)
Eastern Lika		
Capwire TIRM	**147 (125-209)**	**165 (143-227)**
Huggins	168 (110-221)	186 (128-239)

When mark-recapture estimates are done for the entire area without taking into account different intensities of sampling, the models seem to considerably underestimate population size (Table 12, models Capwire TIRM and MhChao). The Huggins model (which includes the area information in the same model) and the composite estimate with Capwire TIRM for Eastern Lika and MhChao for the rest of Croatia are a better fit fort the data, and provide very similar results.

Table 13: Mark-recapture estimates of abundance of brown bears in 2015 in Croatia. The model that we believe to be the best is in bold print. The minimum yearly estimate is the end-of-the-year abundance for 2015, after mortality and just before reproduction (in early 2016). It is the direct mark-recapture estimate minus the animals that died during sampling. The maximum yearly abundance is abundance in spring 2015, and is the minimum abundance plus all recorded mortality in that year. The maximum abundance is underestimated since it doesn't include undetected mortality.

Statistical Model	Minimum Yearly N (95% CI)	Maximum Yearly N (95% CI)
Capwire TIRM	702 (688-787)	846 (832-931)
MhChao	668 (596-742)	812 (740-886)
Huggins	762 (690-834)	906 (834-978)
MhChao + Capwire TIRM	**793 (702-928)**	**937 (846-1072)**

The estimated sex ratio does not differ much from the sex ratio estimated for the entire study area (Table 11). Both model estimates and direct estimates provide very similar results.

Table 14: Model-derived and directly estimated sex ratio for Croatia. Since males have larger homeranges and more transboundary males may have been sampled, their proportion may be slightly overestimated.

Area	Model	Female	Male
Croatia	Capwire TIRM	0.584	0.416
Croatia	Mh Chao	0.585	0.415
Croatia	**Direct Samples**	**0.582**	**0.418**

Management considerations of genetic mark-recapture population size estimates

Ours is the first estimate of the brown bear abundance and sex ratio in NW Dinaric Mts. based on hard empirical data. As such it represents the best possible foundation for science based conservation and management of this species in the entire area.

But there are other values of this study. For the first time we created a reference point to which any future population size estimates in this area will compare. At the level of the entire study area this is still a "snapshot" of the situation, but in Slovenia, where a similar study was already done in 2007, it already means the start of genetic monitoring. With it we have the first direct estimate of population dynamics in the area - it looks positive for bear conservation, but is also opening a wider discussion about tolerance of bears and future of bear management and conservation. Still, whatever the final decisions will be, they will have the best possible science to lean on - should the decision makers so choose.

Genetic diversity of brown bears in NW Dinaric Mts.

Genetic diversity of brown bears in the study area has already been researched (Kocijan et al., 2011; T Skrbinšek et al., 2012b) so this was not a main goal of the study. Still, since a much larger locus set is used in this study and since genetic diversity indices are an important indicator of a population's genetic "health", we decided to report these here. By locus data are summarized in table 16.

Table 15: Genetic diversity indices for brown bears in NW Dinaric Mts. N = number of samples, He = expected heterozygosity, Ho = observed heterozygosity, A = Allelic diversity, pHWE = p value of a Hardy-Weinberg test. The two loci (U64 and Mu26) which don't fit the Hardy-Weinberg expectations were excluded from the summary statistics. One individual that seems like a direct immigrant in the population was also excluded.

Locus	N	He	Ho	A	pHWE
U03	2016	0.65	0.64	5	0.446
U06	2019	0.66	0.66	7	0.144
U14	2020	0.70	0.67	7	0.250
U16	2021	0.75	0.75	12	0.099
U17	2021	0.66	0.66	4	0.904
U25	2021	0.60	0.59	8	0.045
U51	2016	0.77	0.74	11	0.000
U57	2021	0.55	0.54	4	0.457
U63	2019	0.71	0.69	6	0.163
U64	2005	0.57	0.53	7	<0.001
U65	2021	0.65	0.65	6	0.507
U67	2021	0.58	0.57	4	0.097
U68	2016	0.83	0.82	15	0.005
G10B	2018	0.70	0.69	9	0.010
G10C	2019	0.75	0.72	11	0.001
G10D	2020	0.80	0.79	7	0.077
G10L	2019	0.62	0.59	6	0.024
G10P	2018	0.82	0.81	11	0.021
G10X	2019	0.86	0.84	11	0.004
G1A	2013	0.66	0.66	6	0.941
Mu05	2020	0.72	0.72	8	0.521
Mu09	2007	0.74	0.73	11	0.038
Mu11	2021	0.75	0.75	8	0.254
Mu15	2021	0.76	0.75	7	0.010
Mu23	2014	0.80	0.80	9	0.043
Mu26	1952	0.63	0.46	6	<0.001
Mu50	2020	0.80	0.82	7	0.110
Mu51	2020	0.62	0.60	6	0.004
Mu59	2019	0.86	0.85	10	0.270
Summary	2021	0.72	0.71	8.00	

Detection of possible immigrants and population structure

We didn't explore this topic in detail, but looked at the data with a simple principle component analysis (PCA) to detect possible genetic structure and/or immigrants in the population which could affect the findings.

Interestingly, one individual stands completely out (CF.0KU5, figure 18). Looking at the metadata for this sample we didn't find anything special – a male, 120 kg, shot in Croatia in December 2008. We will explore this issue further, but for the purpose of the analyses presented here, the sample was removed.

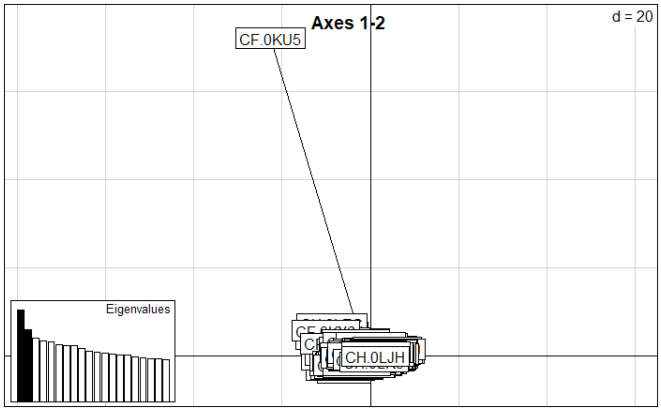

Figure 18: PCA plot with the possible migrant in the population. This issue needs to be researched further.

The basic exploration of population structure indicated some spatial pattern in genotypes, but no clear structure that could affect the downstream analyses (Figure 19). The spatial pattern may in fact be caused by the relatedness between animals, which should decrease through space, and the huge number of samples in our dataset.

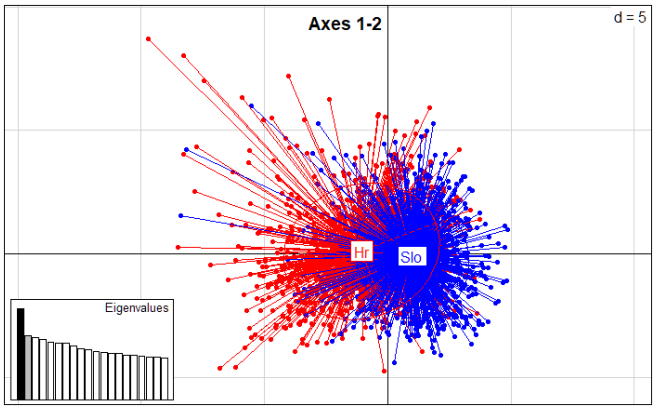

Figure 19: PCA plot of brown bear genotypes in NW Dinaric Mts. Only one component (mapped to x-axis in the graph) seems interpretable. While there is some spatial pattern in genotypes, it shouldn't affect the results and may in fact be caused by the spatial pattern of relatedness since the sample size is huge.

Temporal variation in effective population size of brown bears in NW Dinaric Mts.

Large number of samples and age data allowed us to estimate effective population size (*Ne*) or our brown bear population for 18-year period from 1997 until 2014. For this period we were able to construct large enough samples of animals aged 0-8 years (~ generation interval) to provide comparable estimates of *Ne* for each year. The possible exception of years between 1997 and 2001 where the number of animals from Croatia is small, as is evident from confidence intervals. After year 2014 we don't have adequate sample coverage in all cohorts to provide reliable results.

Table 16: Yearly estimates of effective population size for brown bears in NW Dinaric Mountains in Slovenia and Croatia. Each yearly sample includes animals aged 0-8 years (~ generation interval) alive in that target year. The estimates are not independent, but each estimate in year *t* represents the harmonic mean of *Ne* for the period *t* - GI, where GI is the generation interval (~ 8 years).

Year	N samples	Ne	Parametric CI	Jackknife CI
1997	53	92.7	76.9-115.1	60.0-177.1
1998	75	109.2	93.8-129.6	79.6-164.2
1999	102	139.9	121.5-163.6	107.0-194.4
2000	124	139.7	123.9-159.1	112.1-180.6
2001	177	178.1	160.4-199.0	143.6-228.1
2002	258	156.9	146.0-169.0	135.5-183.5
2003	351	172.8	162.4-184.1	154.0-194.8
2004	413	193.0	181.8-205.1	171.2-218.6
2005	452	210.3	198.7-222.9	186.9-238.0
2006	446	213.8	202.0-226.6	190.4-241.4
2007	457	215.5	203.8-228.3	168.6-282.4
2008	602	225.8	215.0-237.2	180.2-287.2
2009	656	222.2	212.1-232.8	180.2-277.1
2010	697	264.1	251.6-277.4	237.8-294.2
2011	624	236.5	225.2-248.5	207.7-270.6
2012	681	247.2	235.8-259.3	216.9-283.0
2013	581	244.8	232.2-258.5	214.2-281.6
2014	**559**	**261.6**	**247.5-277.0**	**233.0-295.3**

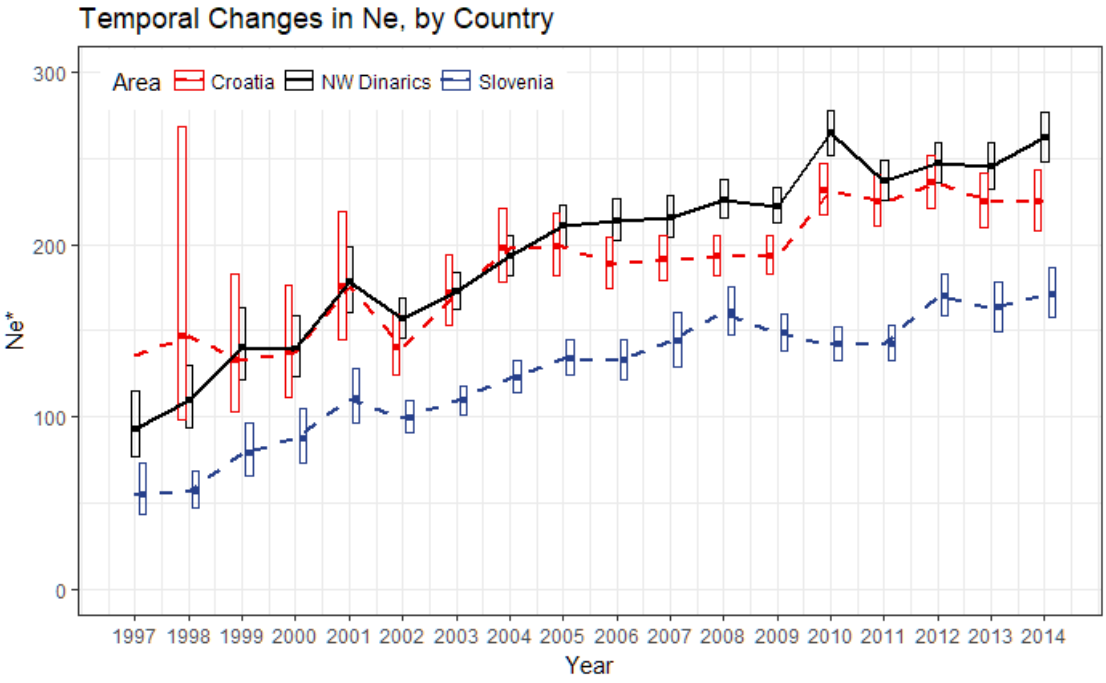

Figure 20: Temporal variation in effective population size of brown bears in NW Dinaric Mts. from 1999 until 2014. Estimates are made using all samples (NW Dinarics) and samples from each respective country. Animals aged 0 to 8 years (~ generation interval) and alive in the target year were used in each yearly sample. The estimates are not independent, but each estimate in year *t* represents the harmonic mean of *Ne* for the period *t* - GI, where GI is generation interval ~ 8 years.

Since an animal is alive for several years, our yearly samples included a very large number of individuals, making the results highly precise. On the other hand the yearly samples must be at least 8 year apart to be fully independent, both because of the sample overlap as well as because the generation *Ne* estimate in year *t* represents the harmonic mean of *Ne* for the period *t* - *GI*, where *GI* is generation interval ~ 8 years.

We see that total effective population size is growing through time (Figure 20), and seems to have more than doubled since the end of 1990s. This is probably the consequence of actual brown bear population growth in this area over the last two decades. The rate of this growth seems to be slowing down in the period after 2005, which could be the result of the considerably increased culling in both countries after 2002.

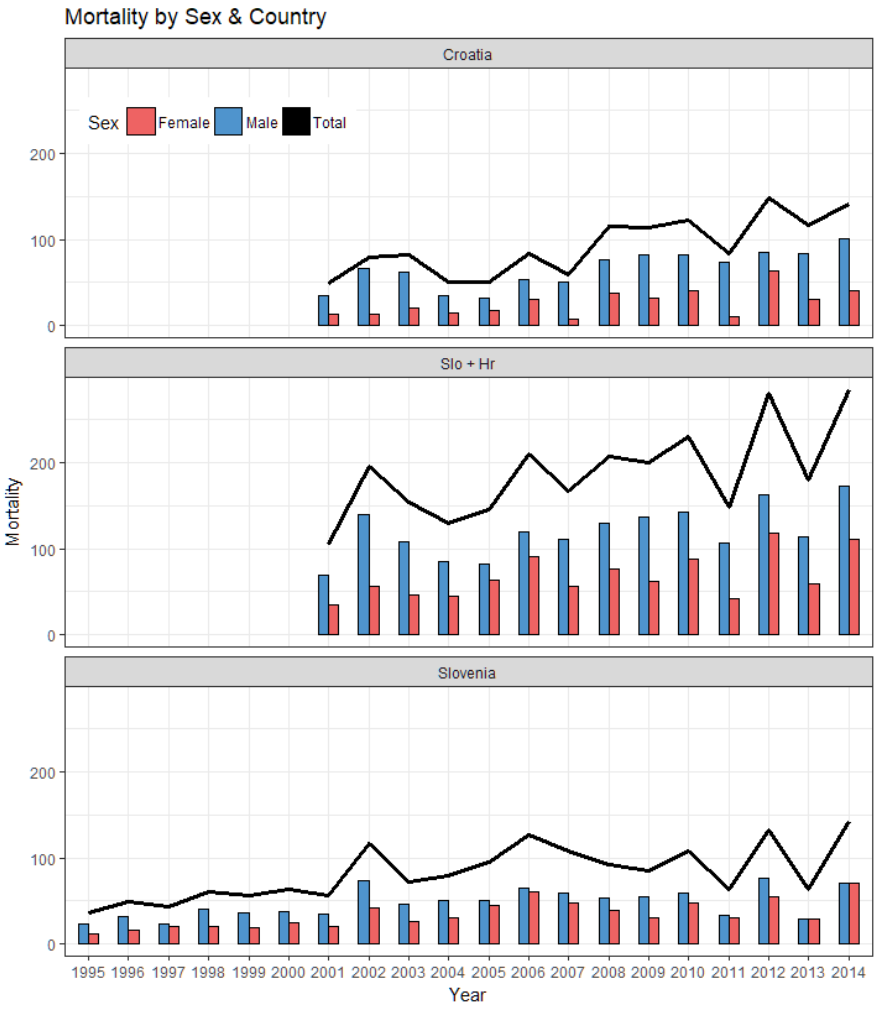

Figure 21: Mortality by sex and country, and cumulative mortality. Data for Croatia for 1990s is lacking, but it is expected that bear mortality at that time was relatively low.

An interesting result is that estimates from each country and both countries together provide different estimates of *Ne* (Figure 20). While estimates using samples from both countries and only samples from Croatia match quite well, the estimates using Slovenian samples are consistently lower. Under the panmixia scenario, where bears are randomly roaming the entire study area, sampling in any specific country (or area) should produce the same results – meaning that all three lines in Figure 20 should be very similar. This is not unfeasible since the entire study area is relatively small and reasonably well connected from the bear perspective, however the actual results deviate from this expectation considerably. Another explanation we could be looking at is a possible cryptic genetic structure, but the resulting Wahlund's effect (mixture LD) would bias the total effective population size low (Robin S Waples, 2014), which we are not seeing. Besides, the Wahlund's effect should cause a genome-wide deviation from the Hardy-Weinberg equilibrium, which does not seem to be the case (Table 14).

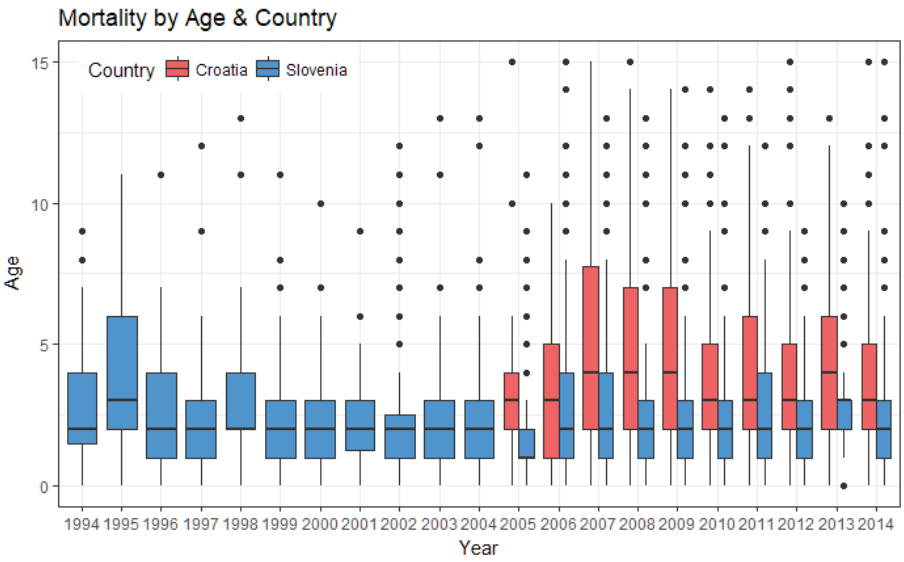

Figure 22: Age structure of bear mortality, by year and country.

An interesting pattern that can explain the observed discrepancy becomes apparent when we look at differences in sex and age structure of mortality in both countries (Figures 21 and 22). While more males are being killed in both countries (which is probably driving the skewed sex ratio we're detecting in the population), this is more pronounced in Croatia. There are also considerable differences in age structure (Figure 22), with the age of killed bears being considerably higher in Croatia than in Slovenia. In Croatia, the structure of cull clearly shows trophy-motivated hunting that targets mainly adult male bears. In Slovenia, on the other hand, cull is strictly prescribed and targets mainly pre-reproductive bears with the intention of regulating population size, with only a small part of the cull quota targeting the large males.

This can cause some interesting effects on genetics of a population (Table 15).

In Slovenia, high mortality of young animals and low mortality of adults limits recruitment into reproductive classes. This causes the same animals to monopolize reproduction, decreasing the pool of parents, increasing variance in lifetime family size (most animals have no offspring, few animals have a lot of offspring) and decreasing effective population size. Also, high mortality in pre-reproductive classes and young animals makes any immigrants from Croatia less likely to succeed, effectively limiting geneflow from Croatia and "localizing" genes of Slovenian bears.

In Croatia on the other hand the trophy hunting approach to bear management causes quite the opposite effect. High mortality of adult males "makes room" for young males to join reproduction. Young females likewise have low mortality, and once they join reproduction they are largely protected when they have young with them. This decreases lifetime variance in family size and increases effective population size. An interesting effect can be expected on geneflow from Slovenia, since young animals from Slovenia have a higher chance of surviving and joining reproduction in Croatia than in Slovenia, causing geneflow into Croatia and "mixing" the genes.

All above would clearly cause the genetic picture we're observing.

Table 17: Main expected drivers of differences in estimates of effective population size in Slovenia and Croatia.

SLOVENIA		
Management approach	**Effect**	**Effect on population genetics**
Strictly regulated culling with intention of regulating population size: - High mortality of young animals. - Low mortality of adult animals	High mortality of young animals and low mortality in reproductive classes drives low recruitment in reproductive classes.	Small number of animals, particularly males, monopolizes reproduction. High variance in lifetime reproductive output. *Decreases Ne*
	Young immigrating animals from Croatia have high probability of mortality and low probability of reproduction (particularly males where reproduction is dominated by large adults).	Genes of Slovenian bears remain "local". Low geneflow from Croatia. Deviation from the panmixia scenario. *Local bias in Ne estimates*
CROATIA		
Management approach	**Effect**	**Effect on population genetics**
Trophy hunting, mainly targeting adult males.	High mortality of adult males drives high recruitment of young males in reproductive classes.	High turnover among reproductive males, lower variance in lifetime reproductive output for males. *Increases Ne*
	Young immigrating animals from Slovenia have a high chance of survival and recruitment into reproductive classes.	High geneflow from Slovenia, high mixing with bears from Slovenia. *Ne estimates with local samples approach population Ne*

Effective population size – conservation and management considerations

We can see that different management decisions taken over a relatively small area can have considerable and rather unexpected effects on the most basic genetic parameters critical for conservation. It's been known and discussed for quite a while that humans can have considerable effect on evolution of species they interact with, which may be particularly true for the wildlife species that are harvested or otherwise managed by lethal means. This is something that will need to be discussed in the future also for our bear population, and taken into account in management planning.

However, this is neither simple nor clear-cut. Having a large effective population size is clearly critical for conservation since it reduces loss of genetic diversity, slows accumulation of inbreeding and provides the "environment" for selection (and hence evolution) to work. In populations with low *Ne* (the rule-of-the-thumb is below *Ne* = 50) the decision is quite simple - increasing this parameter will be critical for the population to survive at all, and extinction is the most definite killer of any evolutionary potential. However, as soon as *Ne* is high enough for this not to be a problem, not all means of increasing effective population size are necessarily beneficial for a population or a species from the evolutionary perspective. Effective population size of the brown bear population in NW Dinaric Mts. is not small (the 2014 estimate is 261.6 (247-277)). This is enough to avoid inbreeding, but still shy of the rule-of-the-thumb 500 threshold considered important for preserving evolutionary potential. This certainly emphasizes the importance of allowing and promoting connectivity towards other bear populations in the wider region, but doesn't necessarily mean that effective population size in NW Dinaric Mountains should be increased by other management changes as a matter-of-fact.

The Croatian management model has the advantage of increasing *Ne.* Trophy hunting also increases value of bears for hunters as one of the most important stakeholder groups, and provides direct economic benefit. All this can result in a higher tolerance of bears. On the other hand trophy hunting increases skew in sex ratio and removes the least problematic animals from the population (although some research (Elfström et al., 2014) indicates that presence of large dominant males can drive young males and females with cubs closer to humans, increasing conflicts). Also, trophy hunting may have the effect of removing the most successful individuals and possibly negatively impacting the evolution of the species.

The Slovenian management model has the advantage of conserving the "reproductive core" of the population, limiting possible negative effects of management decisions. It also conserves the most successful animals, possibly benefiting evolution of the species. It removes the animal categories that often get into conflicts with humans, although it can create the "despotic bears" situation (Elfström et al., 2014) and increase the actual conflicts. On the other hand it decreases effective population size and increases loss of genetic diversity. Also, it decreases the value of bears for hunters and decreases economic benefits, possibly decreasing the critical factor for bear conservation in our landscapes: the human tolerance.

Both management models have their pros and cons, and a wider debate should be started about how to include this new understanding into practical bear conservation and management.

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