LIFE DINALP BEAR

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

LIFE13 NAT/SI/000550

IMPLEMENTING ROBUST AND COST-EFFECTIVE GENETIC MONITORING OF BROWN BEAR POPULATION SIZE

WORKSHOP REPORT AND BEST PRACTICE RECOMMENDATIONS

Action F.2: Networking with other LIFE and/or non-LIFE projects

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December 2018

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Summary

Noninvasive genetic sampling paired with mark-recapture modelling has proven several times to be currently the best available method for estimating the size of bear populations in the wild. In recent years, huge strides in DNA sequencing techniques opened new and exciting opportunities also in the field of wildlife monitoring and noninvasive genetic sampling. Extensive development is also ongoing in the field of mark-recapture analysis, enabling increasingly robust data analysis. However, such studies remain difficult to implement in practice, and there are many pitfalls that a researcher should be aware of.

A workshop "*Implementing robust and cost-effective genetic monitoring of brown bear population size*" was held at the 26th International Conference on Bear Research & Management titled "Life With Bears" in Ljubljana, Slovenia between 16th and 21st September 2018. The workshop covered the state of the art in laboratory and data analysis methods and investigated the critical issues that need to be considered in study design and organization of fieldwork so that data/samples are collected in a manner that facilitates further analyses. Based on the presentations and discussion at the workshop, we prepared the best practice guidelines that briefly summarize the state of the art and new developments in genetic monitoring of brown bear population size.

Workshop, discussion and production of this document

The main goal of the workshop was to discuss the state of the art in genetic monitoring of brown bear population size. Specific goals were: 1) to discuss available laboratory and data analysis methods and their relative advantages/disadvantages, 2) to share ideas and experiences in study design and organization of fieldwork, and 3) discuss specific issues / ideas that participants have for similar studies they might be planning. We 4) summarized the conclusions in Recommendations for genetic monitoring of brown bear population size (this document).

Both keynote speakers presented their view on the state of the art of genetic monitoring of brown bear population size and illustrated this with personal experience and recent studies. A lively discussion was started after the presentations. Following both the messages from the keynote talks and the discussion that followed, the hosts compiled this document of best practice recommendations for anyone considering genetic monitoring of size of a brown bear population.

Designing a study

Although sampling design is always important, it is seldom as critical as in the case of capture-mark-recapture (CMR) studies, especially if the research goal is an abundance estimate. CMR modeling is becoming extremely flexible through development of new models and software packages, but all this becomes useless unless the data has not been collected in a manner that satisfies the modeling assumptions as much as possible. In a poorly designed study, it can easily happen that the researcher will, at the end of the day, not be able to say anything about the population size beyond the minimum, the number of different animals detected in the sampling. While this my be sufficient in some cases, ambitions are usually higher and such a result may often be considered a failure.

There is one simple rule for study design – there is no universal study design appropriate for all areas and all circumstances. Each study will be unique as researchers will need to balance their ambitions with their field reality, and reality of their financial and human resources. This will guide the general design of the field effort, and consequently of all downstream analyses, from laboratory to data analysis and reporting of results.

Still, there are a couple of guidelines that can help design a feasible study that will have a good chance of success.

1. **Understand what you want to do and the assumptions of the CMR models you are likely to use <u>before</u> you go out collecting samples.**

This point can't be overstressed. As much as it sounds logical, it is very tempting to go out and start collecting samples as soon as possible, especially with tight deadlines looming over the horizon. This is a dangerous trap, as the sample collection is usually the only part of the research that is impossible to repeat. In other words, make sure you get it right the first time around, as there will most likely be no second chances.

Besides understanding the general requirements of mark-recapture studies, the rapid methodological development over the last decade provided researchers with a (relatively) new approach that allows for direct estimate of population density and correction of the edge effect (modified capture probability caused by parts of animals' ranges being outside of the study area). This is **Spatially Explicit Capture Recapture (SECR)**, which is a very interesting and promising development. In the workshop discussion we came to the general conclusion that SECR should be used whenever the study design feasible in the field allows it. However, this does require the researcher to get familiar with these methods before designing fieldwork, so that the design meets the assumptions and requirements of the appropriate SECR models as much as possible. Also, not all sampling schemes are currently amenable to SECR, but this may change in the future.

Mark-recapture can be used to estimate a number of parameters, not only abundance. Make sure you understand all the goals of your study and adjust the design appropriately so they can be reached.

2. Choose your study area and timing of the field season wisely

With classic CMR studies, you typically want to use the models for demographically closed populations since they are statistically more powerful. This means that you should satisfy (as much as humanly possible) the assumption of population closure. If your circumstances allow you to use SECR, this requirement (at least its spatial component) can be relaxed.

Spatial population closure you can obtain by carefully selecting your study area. Ideally, you would sample the entire range of a population, but this is often impossible in practice in large or even medium sized populations. If only a part of the range is sampled, there are a couple of tools available to minimize the violation of closure assumption:

- **Shorten the sampling**. Make the sampling as short as possible to decrease the chance of bears moving in and out of the study area.

- **Use geography to designate the study area**. Try to designate the area so that it's delimited by some landscape features that are difficult for bears to cross (river, road, high mountain ridges…).

- **Use species' biology**. Bears don't move around with equal intensity year round. For example, in Europe it makes sense to sample brown bear scats in autumn since bears are in their hyperphagia period, consume a lot of food (and hence produce a lot of samples) and tend to move around less.

- **Don't sample across the period when new cubs are being born**, unless you wish to study fecundity – but in this case the entire study should be designed with this in mind.

There are a few general sampling designs out there, each of them with its strengths and its weaknesses. While there are many details a researcher needs to study and decide before organizing a field season, we will stay general.

- **Collection of hair samples** using a grid of hair traps. While the first large genetic estimates of bear abundance were done using this type of design, it is still the best (if not only) choice when inaccessible areas are sampled. Its main benefits are good spatial distribution of sampling effort and the possibility to set up hair traps in areas accessible only by considerable effort or expenses (long hiking to areas accessible only on foot, helicopter…). This sampling design is also ideal for SECR as the method has been originally developed for trapping grids. There are also several weaknesses. One is that the hair traps require some participation on the side of the bear, which can create either behavioral responses (trap happy / trap shy), or capture can be sex or age biased. Also, setting a hair trap requires some costs and effort, and usually requires professional field personnel (which can be augmented with volunteers), which means that costs of such a sampling design can be high. An interesting twist on this sampling design is use of rub trees (trees that bears naturally use for marking) or wooden power poles (which bears use as rub trees).

- **Collection of scat samples**. The main strength of scat sampling is simplicity. Practically anyone can be taught to collect a scat sample, and very large studies can be done using a large network of volunteers in a "citizen science" approach in a very cost-effective manner. Scat samples are also logically less biased by sex and age and their collection shouldn't create a behavioral response, although bear cubs can still be underrepresented. While genotyping success rates can be low if all found scats are collected and analyzed, they can be very high if only fresh scats are sampled. On the downside, sampling scat samples can be difficult if the terrain is inaccessible. Also, it is currently difficult to use SECR unless sampling effort is recorded in a spatial manner – feasible with professional field personnel, very difficult with volunteers.

- **Both hair and scat** can be collected in a study, capitalizing on strengths of both sampling designs.

3. Estimate the amount of field effort required

Sampling is expensive and time consuming. On the other hand, you need enough samples to get a results. So, how many samples does one need to obtain useful estimates? A rule of the thumb, suggested by Solberg et al. (2006), is to aim at collecting 2.5—3 times the number of samples as is the "assumed" number of animals present in the researched population if the goal is an abundance estimate. A better understanding of the required effort can be achieved with a power analysis. For "classic" CMR one can use MARK (White & Burnham 1999) simulation models. Several sampling scenarios can be simulated, and the results analyzed to understand what confidence intervals to expect from a certain number of successfully genotyped samples. A thing to consider is the expected genotyping success rate, which should be used to correct the estimated number of required samples. While genotyping success rate close to 90% were obtained from scat samples when only fresh samples were collected, a more conservative estimate of 60-65% should be used for planning if no experience of noninvasive genotyping from the planned study area exists.

Making the field season work

Any medium or large study will require considerable amount of preparatory work to organize a successful field season. A researcher should ideally start making plans at least 6 months, even better a year before the actual sampling. Logistics of large studies can be overwhelming.

One thing that should be done if possible is to **keep track of the progress of fieldwork** as it happens. This means a prompt entry of data into a database as samples are being collected, and analysis in regular intervals (up to 1 week) in a geographic information system (GIS) to determine spatial and temporal distribution of the collected samples. In this manner deficiencies (e.g. poor spatial coverage) can be detected in a timely manner and corrected.

If working with volunteers, either as "citizen science" or including them in efforts directly managed by field personnel, there are a couple of pointers to keep in mind:

- **Start organizing and advertising early.** People need to know that they can participate in studies, and to have enough time to plan and organize their life. A couple of months at least.

- **Make participation simple.** If getting involved requires a huge effort or even expense on the side of volunteers, there is a chance that they won't decide to join. Make all sampling materials easily available to the participants. Provide clear instructions. Make sending the samples to the laboratory as easy as possible for the participants.

- **Provide feedback!** This cannot be overstated. Volunteers don't work for free, they just don't get money for their work. Their reward is experience, sense of accomplishment… each person will have his or her own motivation, but all of them will be curious about the impact their work had.

Laboratory analyses

Outsourcing the analysis to a competent laboratory

There are now many competent commercial and academic laboratories that can handle noninvasive genetic samples. However, genetic laboratories come in many flavors, and not all of them will have sufficient equipment, resources and know-how to handle noninvasive genetic samples. Some pointers:

- **Ask about references**. If the laboratory has extensive experience in handling noninvasive genetic samples, doing forensic genetics, or handling historic or environmental samples, there is a good chance that laboratory procedures will be good enough to obtain high-quality data. If not, rather look elsewhere.

- **Ask about laboratory organization and contamination prevention** (see below). Are laboratories for DNA extraction from noninvasive genetic samples and other pre-PCR procedures physically separated from PCR and post-PCR work? Are pipetting tips with aerosol filters used for all liquid transfers? Ask about other contamination prevention procedures in the laboratory. This is essential.

- **Ask about error handling and genotype quality assurance.** How many repeats will a sample have, what is the procedure for accepting/rejecting a genotype?

If a laboratory doesn't have experience in handling material with low DNA quantity and quality, there are good chances that something will go wrong.

Doing analyses "in house"

Many organizations typically involved in genetic monitoring of brown bears will have their own genetic laboratory that may or may not have experience with handling noninvasive genetic samples. There are several issues to keep in mind.

Noninvasive genetic samples have a minute quantity of target DNA of poor quality. This narrows down the main problems into two categories: contamination prevention and error handling. Of course the laboratory should also have well-established protocols for tracking of samples (preferably automatic with barcodes or RFID) and results (laboratory databases), which becomes particularly important in medium to large studies (over approximately 500 – 1000 collected samples).

Laboratory organization

We will describe laboratory organization at University of Ljubljana where we have considerable experience with analyzing noninvasive genetic samples. Many other laboratories that we collaborate with are organized in a similar manner.

We have two physically separated laboratories for noninvasive samples and for tissue samples, a separate PCR room and a separated post PCR / sequencing facility. We're enforcing strict regimes regarding movement of personnel, equipment and material between laboratories to prevent contamination. Laboratory for noninvasive samples is used for extraction of DNA from noninvasive and historic samples, and for PCR setup from such material. For tissue samples, PCR setup is done in the tissue laboratory under a pressurized PCR hood. All flow of material during analysis is one way, meaning that once any material leaves the room where material with low DNA concentration was handled, it never returns (e.g. PCR products are never returned

into the tissue lab, or anything from the tissue lab is never brought into the noninvasive lab). In the noninvasive lab these procedures ware especially strict. In that lab, we also limit the movement of personnel, with a rule that anyone that was in any of the rooms where higher concentrations of DNA were handled (tissue lab, PCR room, sequencer room) has no entry into the noninvasive laboratory until they have taken a shower and changed their clothes. All working surfaces in all laboratories are regularly (usually daily when laboratories are being used) decontaminated with 10% sodium hypochlorite (bleach).

Good laboratory practice and training of personnel

Noninvasive genetic samples are less forgiving of sloppy labwork than samples with good DNA. If a laboratory is considering analyzing such material for the first time, it is a good idea to send someone for training in a well-established laboratory that has extensive experience in analyzing noninvasive genetic samples. In a laboratory that has a lot of experience with such material, any person new to the laboratory, even a student working on a diploma thesis, should be trained and supervised until he or she is capable of independent work. A single poorly trained person can create considerable problems for the entire lab (e.g. contamination of pipettes or stock primers).

Pipetting tips with aerosol barrier filters should be used throughout. To minimize possibilities of sample mixup, sampling arrangements should be photographed or filmed for every PCR setup, or a pipetting robot should be used. Critical points in the analysis process should be determined, and routine checks established to catch errors. Pipettes should be decontaminated and calibrated in regular intervals, and never used outside of the laboratory. The same goes for all other labware and instruments.

Assuring genotype reliability and error handling

There is now a large body of literature available regarding genotyping errors and how to get around them, and anyone starting with analysis of noninvasive genetic samples should make an extensive study. A few pointers:

- With noninvasive genetic samples, genotyping errors are a fact of life. They cannot be avoided, they must be handled.

- Analysis of all noninvasive genetic samples should be done in several parallels, with the “multiple-tube” approach. A consensus genotype should be done, and reliability of such a genotype assessed through statistics.

- Not all samples will produce a reliable genotype, but some of them may come close. Resist the temptation of using poor genotypes that you can’t confirm through some other samples of the same animal for downstream analyses. Such data should be removed – no data is better than wrong data.

- Even with tissue samples, at least two parallels should be done, or at the very least a random sample (~20% or more) should be re-analyzed and the residual error in the dataset assessed.

Data analysis and capture mark recapture (CMR)

Capture – mark – recapture (CMR) analysis is a field of its own, and much too extensive to go into here. We'll provide a few pointers.

- **Study the CMR methods <u>before</u> sampling**. This was stated before, but an understanding of CMR is critical in study design. The data from a poorly designed study may be useless for CMR analysis, or at best provide poor results.

- **Make sure you understand the assumptions of the methods you're using**. It's easy to plug the numbers in a software package and get some results. However, without an understanding of the assumptions of the underlying methods and the fit of your data to these assumptions the results can be severely biased. Since results of genetic estimates of brown bear abundance are often used for practical management, wrong results can have serious unintended consequences.

- **If unsure, ask an expert in the field for help**. Preferably during the study design phase, as in the data analysis phase it can already be too late!

Perspectives and conclusions

Genetics is one of the fastest evolving and diversifying fields of science. Explosive methodological development is followed by an increase in speed and reliability of analyses, and a decrease in prices. Parallel to that is the increase in computing power, and development of data science. All this will affect the way we conduct genetic abundance estimates of brown bears. We will mention some of the developments that may have the most impact.

- **New approaches to genotyping**. A very interesting approach has been recently proposed for genotyping of noninvasive brown bear samples. The method uses genotyping-by-sequencing of microsatellites on a high-throughput sequencing platform. This speeds-up the analyses by an order of magnitude and decreases associated work costs, paving the way for very large, cost effective studies. The resulting genotypes, being direct DNA sequences, are completely transferrable between laboratories and independent of the instruments used to produce them, making them future-proof and open for wide collaboration. The method has already been used in a large brown bear population abundance estimate in Slovenia and Croatia and proven to be effective.

- **Spatially Explicit Capture Recapture (SECR)**. This relatively recent take on the capture-mark-recapture solves some of the problems that have been plaguing such studies since their inception, particularly when abundance and population density are being estimated with only a part of a population range sampled. If a study can be designed and conducted to conform to the current SECR requirements, it should use SECR. Although not all sampling designs that make sense from the field perspective can currently accommodate SECR, we can expect these methods to further develop in the future.

Genetic studies of brow bear abundance are in many populations already becoming the “golden standard” of monitoring. In most cases, they are the only scientifically defensible way of estimating abundance of a population in the wild. They usually require considerable financial and human resources but are often warranted to help overcoming the challenges faced in conservation and management of brown bears world wide. With rapid methodological developments, faster analyses and decreasing laboratory costs, we can expect genetic abundance estimates of bear populations to become even more common in years to come.