ANALYSIS OF NONINVASIVE GENETIC SAMPLES FROM BROWN BEARS (*Ursus Arctos*) FROM THE TRANSBOUNDARY PRESPA BASIN

Report

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December 2021

Acknowledgements

We gratefully acknowledge the contribution of the PrespaNet partners – MES, PPNEA and the SPP – and all the volunteers who helped with the fieldwork and the collection of samples. In addition, we also acknowledge invaluable support from the Management Body for the Prespa National Park (Greece), the Prespa National Park (Albania), Galicica National Park, Pelister National Park, the Municipality of Resen (Ezerani Nature Park) and the volunteer students from Fan S. Noli University in Korça.

In Albania: Bledi Hoxha, Aleksander Trajce, Olsion Lama, Ilir Shyti, Cveta Trajce and Klaudja Koci

In Greece: Olga Alexandrou, Giorgos Catsadorakis, Julia Henderson, Myrsini Malakou, Haris Nikolaou, Lazaros Nikolaou, Irene Koutseri, Georgia Reppa, Konstantinos Papadopoulos, Thanos Kastritis, Christina Ninou, Ilias Fotakis, Vasiliki Faki, Annie Anastasiadou, Vasilis Papadopoulos, Fyllio Nitsopoulou, Eleana Kazila, Nikos Giannakis, Angela Georganda, Sinead Corcoran, Victoria Saravia, Panagiotis Valatidis, Julian Hoffman, Kyriakos Poukamisas, Nikos Tsilis

In North Macedonia: Daniela Zaec, Aleksandar Pavlov, Dime Melovski, Aleksandar Stojanov, Dragan Arsovski, Jonche Gagovski, Dejan Dimidzievski, Laze Uzhanovski, Sara Krsteska, Andrej Mihajlov, Jovan Vuković

Lastly, we would like to express our gratitude to the Prespa Ohrid Nature Trust and the Aage V. Jensen Charity Foundation for their generous financial support for these activities within the project "Strengthening NGO-led Conservation in the Transboundary Prespa Basin", carried out by the SPP, MES, PPNEA and EuroNatur.

Summary

Estimating the abundance of brown bears in the wild is a daunting task, but the possibilities opened-up considerably with development of molecular genetics and non-invasive genetic sampling. This allows use of capture-mark-recapture methods, and robust abundance estimates also in wild populations. However, these methods require a lot of field effort and considerable laboratory and analytical expertise, which often makes them costly and difficult to apply.

This study was conducted in Prespa, a biodiversity hotspot shared by Albania, Greece and North Macedonia. The goal of the study was to get a basic idea about brown bear abundance in the area, with the secondary goal to get empirical baseline data on the genetic diversity of these bears. It is part of the project "Strengthening NGO-led Conservation in the Transboundary Prespa Basin", implemented by the PrespaNet NGO network.

We used noninvasive genetic sampling, and next generation DNA sequencing to analyze the collected samples of brown bears. The samples were collected within a two-year concerted effort in 2018 and 2019 by the project partners. The DNA was extracted in a laboratory dedicated to historic and noninvasive genetic samples, and each sample was amplified with primers for 13 microsatellite markers and a sex-ID marker, labelled with DNA tags on F and R markers to enable sample identification. PCR products were pooled, and the resulting library was sequenced on an Illumina HiSeq sequencer. The sequence data was bioinformatically analyzed to obtained genotypes and sex ID for each sample. We routinely repeated the entire analysis 8 times, and then another 8 times for all samples that we were not able to genotype in the first 8 repeats, but that provided any bear-specific PCR products, so that effectively the analysis of each sample was repeated 8 to 16 times. Genotypes were matched with each other to identify which samples belonged to the same individual animal, and the basic parameters of genetic diversity were calculated and compared with other brown bear populations in the region.

Altogether we collected and analyzed 227 scat samples: 42 from Albania, 113 from Greece and 69 from North Macedonia. We successfully genotyped 118 (52%) of the samples, with the success rate varying considerably between countries and collection years. The success rate was less than ideal, and we managed to track the problems to the field collection of old samples, some inappropriate sample storage material, and long (and possibly inadequate) sample storage for some samples before analysis.

We detected 51 different bears, 19 females and 32 males, distribution is shown in Figure A. This represents the minimum number of animals in the area at the time of the study. Sampling intensity and genotyping success rate were too low to yield enough recaptures to allow population abundance estimates with capture – mark – recapture modelling. The difference in the number of detected males and females is considerable, but we believe that this doesn't reflect the actual sex ratio in the population but is rather the result of study design and differences in space use between sexes.

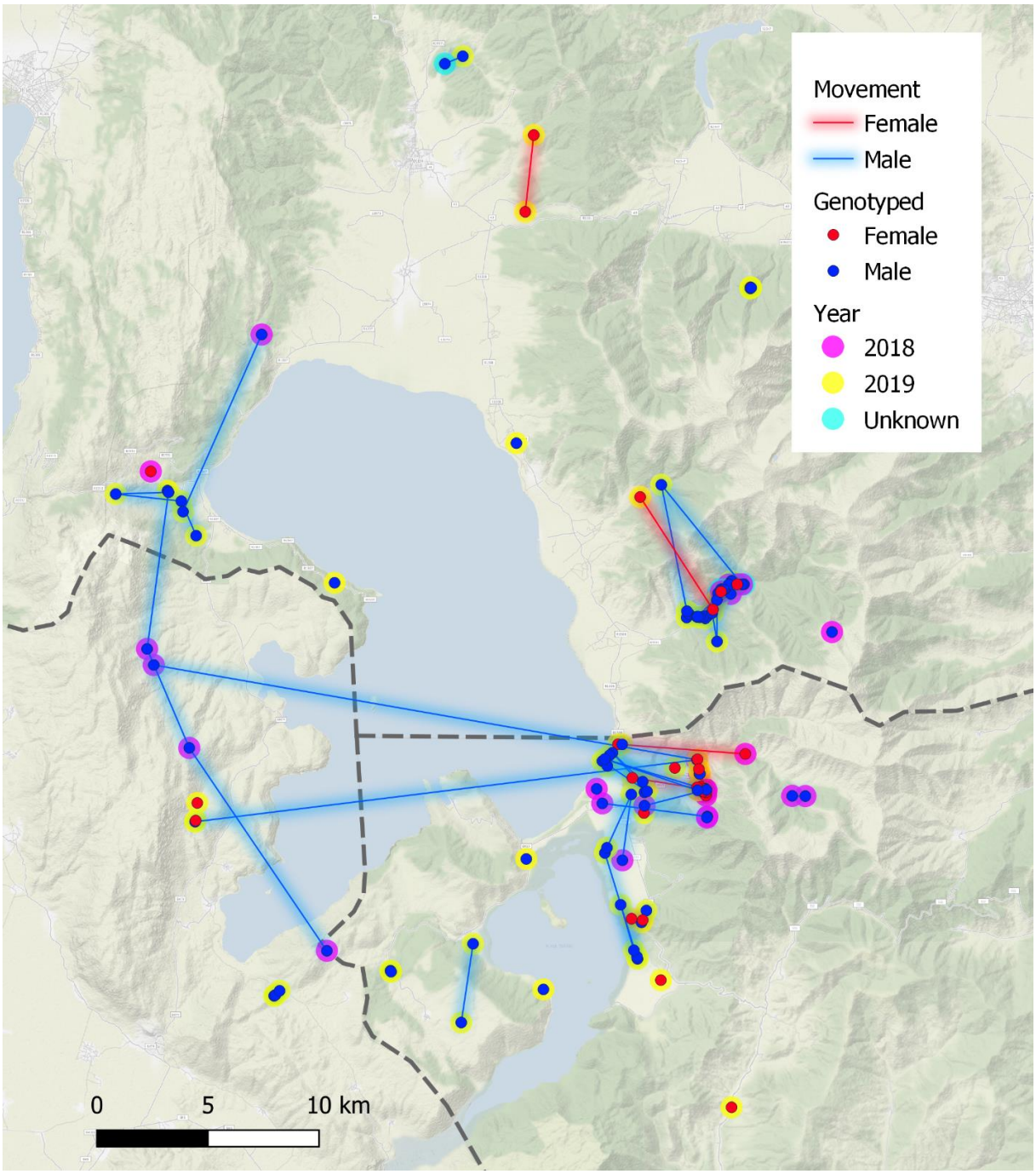

Figure A: Samples and genotyping results. Lines connect the samples of the same animal.

Genetic diversity in bears in the Prespa Region seems somewhat lower than that observed towards the west of the Balkans (Table A). This fits with the findings of other studies in that wider geographic region, however the genetic diversity is still considerably higher than in the European bear populations that are known to be very small and endangered (Apennine and Cantabrian populations).

Table A: Genetic diversity indices. N = number of individuals genotyped, He = expected heterozygosity, Ho = observed heterozygosity, A = allelic diversity. SE = standard error. The same markers were used in all studies.

Area	*N*	*He*	*SE He*	*Ho*	*SE Ho*	*A*	*SE A*
Prespa Region	**51**	**0.566**	**0.050**	**0.541**	**0.055**	**4.923**	**0.512**
Montenegro	53	0.649	0.020	0.630	0.021	6.077	0.560
BIH	67	0.638	0.023	0.616	0.023	6.000	0.506
Croatia - Lika E	86	0.659	0.026	0.638	0.028	5.692	0.458
Croatia - Lika W	119	0.671	0.024	0.685	0.021	5.923	0.560
Croatia - Gorski kotar	351	0.665	0.025	0.680	0.024	5.923	0.560
Slovenia	491	0.662	0.022	0.672	0.022	6.000	0.588

The genotyping success in this study is within the bounds that can be reasonably expected in a study that uses noninvasive genetic samples, and perfectly acceptable for a pilot study, but it is not as good as we hoped it would be. However, we did manage to identify many of the issues that decreased the study genotyping success, they are all easily addressed, and we provided concrete recommendations for improvements in this report. This allows for considerable optimism if a similar or larger study would be repeated and, considering all the field know-how and experience that was gained by the project partners, we firmly believe that a highly successful study of brown bear population size and distribution could be done in the area in the future.

Contents

Introduction

Estimating the abundance of brown bears in the wild is a daunting task. In practically all cases we can't directly observe the bears in a manner that we could use to estimate their abundance. Tagging them with individual specific tags is highly demanding and unfeasible in any real-life setting, as is reading of such tags.

The possibilities opened up considerably with development of molecular genetics and non-invasive genetic sampling, which allowed individual "tagging" of bears through genotypes obtained from "noninvasive" genetic material that they left in the environment (feces, hair, etc.). This allows use of capture-mark-recapture methods, and robust abundance estimates also in wild populations. However, these methods require a lot of field effort and considerable laboratory and analytical expertise, which often makes them costly and difficult to apply, particularly in countries with lower GDP.

Because of that, in many cases the abundance estimates of brown bear populations are still based on expert opinions and rarely backed by any defensible methodology and data. This is frequently followed by the often-justified skepticism in the validity of such estimates, and a large spectrum of interpretations and "abundance estimates" by various experts that depend more on the attitude and the stakeholder group of the person doing the estimation than on any facts or reality. Since, invariably, some of these estimates end up being used for management decisions, such decisions are often challenged by different stakeholder groups and difficult to defend. This can lead to a toxic situation where bear management ends up being based on a political tug-o-war that has little connection with the actual situation and conservation needs.

This study was conducted in Prespa, a biodiversity hotspot shared by Albania, Greece and North Macedonia. It is part of the project "Strengthening NGO-led Conservation in the Transboundary Prespa Basin", implemented by the PrespaNet NGO network. This NGO network consists of three partners, the Macedonian Ecological Society (MES), the Protection and Preservation of Natural Environment in Albania (PPNEA) and the Society for the Protection of Prespa (SPP) from Greece.

The broader scope of the study was to estimate the dietary habits of the brown bear and use the fresh samples to genotype the different individuals. The results will create the basis upon which sound conservation measures would be designed.

The study was done using noninvasive genetic sampling. While the goal was to get some idea about brown bear abundance in the area, the secondary goal was also to get empirical data on the genetic diversity of these bears.

Previous efforts were made to estimate the size of the population in the region and the most recent being from 2012 (Stojanov et al. 2012), which uses questionnaire surveys to guesstimate the population number of the brown bear in the wider Prespa region to be around 60, of those 35 in Macedonian Prespa, 18 in Greek Prespa and 7 in Albanian Prespa.

Methods

We used noninvasive genetic sampling and next generation DNA sequencing to analyze noninvasive genetic samples of brown bears.

The methods are state of the art. 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.

Samples

The samples were collected in the Transboundary Prespa Basin, in concert with the ongoing two-year effort of collecting scats from brown bears for estimating their dietary habits (Gonev et al., in prep.). The majority of samples used for the genetic analysis were collected between September and December of 2018 and 2019, although the whole collection period lasted from 11 January 2018 until 13 December 2019. The samples were transported to the Biotechnical Faculty, University of Ljubljana laboratory in Slovenia for analysis. Altogether we received and analyzed 227 scat samples: 42 from Albania, 113 from Greece and 69 from North Macedonia. There were 15 samples from North Macedonia without a recorded collection date and we were able to genotype 8 of them. There were also 4 samples without recorded geographic location, 3 of them successfully genotyped. For three samples we didn't receive any data – we genotyped them (since the data about samples was received after the samples), but none of them provided a useful genotype and were discarded from further analyses.

Upon reception of the samples, we noticed that many of the tubes used for sample collection were inappropriate. Most tubes in samples from Albania were leaking, and the same problem (but to a lesser degree) was with tubes from N. Macedonia. Leakage was not a problem for contamination since tubes were cleaned before samples were taken out for DNA extraction, but in many cases it was difficult to read the sample ID, and many samples were "dry" (the ethanol leaked out), which is bad for conservation of DNA in such a sample.

Sample handling and storage, DNA extraction

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 transferable 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. We're using a relatively new method of genotyping microsatellites using next-generation sequencing, 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, with results that are completely transferable between laboratories and hence "future-proof".

Sample handling and storage

When received in the laboratory, each noninvasive sample received a unique barcode to completely avoid manual labeling 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 in the laboratory database.

Noninvasive samples were 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.

We keep a genetic bank of all collected genetic material of brown bears. All DNA extracts are organized, registered in the database, and tracked using barcodes.

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 have 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.

Figure 1: Inside of the Hamilton Starlet liquid-handling robot in our laboratory during DNA extraction.

We're using a liquid handling robot (Hamilton Starlet) for 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. The extraction is done using the MagMAX DNA Multi-sample Kit (Thermo Fisher Scientific), with a protocol modified for noninvasive genetic samples.

Genotyping

We're using the method described by De Barba et al. (2017) 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.

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 PCR Purification Kit (Qiagen), quantified on a Qubit fluorometer (Thermo Fisher Scientific) and sequenced on an Illumina HiSeq sequencer, resulting in approximately 10 million DNA sequence reads per library.

Once the sequences are received (basically a very 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. (2017), 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.

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

Since the quality of samples was relatively low, we repeated genotyping of any sample that provided specific PCR products, but we couldn't reliably genotype it in the first 8 repeats. We did 8 additional repeats, so we analyzed many samples up to 16 times.

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 analyzed 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 strictest quality assurance protocols, are unavoidable in noninvasive samples (Taberlet et al. 1999; Waits & Paetkau 2005). Incorrect matching can cause considerable biases in mark-recapture estimates (Roon et al. 2005). A solution has been proposed to analyze the minimum number of loci that still provide enough resolution to reliably 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 analyzed the errors caused by allelic dropout remain a significant issue. In such cases, many 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 number 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, such PID or PIDsib, however, 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 analyzing a relatively 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 (Skrbinšek et al. 2012) 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.

Exploring basic population genetics parameters

Population genetics parameters of the Dinaric-Pindos brown bear population are quite well explored in Slovenia and Croatia (Skrbinšek et al., 2012, Skrbinšek et al. 2017), there was a study done in Bosnia and Herzegovina and Montenegro (Skrbinšek et al. 2020) and studies have been done in Greece (Karamanlidis et al. 2017) and North Macedonia (Karamanlidis et al. 2014). We calculated basic genetic diversity indices (observed and expected heterozygosity, allelic diversity) and compared them with the

results of the studies mentioned above that used the same markers in Slovenia, Croatia, Bosnia and Herzegovina and Montenegro. We used R and package ‘adegenet’ (Jombart et al. 2009) for these analyses.

Spatial visualization

Samples and genotypes were visualized (mapped) using QGIS 3.20.

Results

Genotyping success

We managed to successfully genotype a total of 118 samples (52.0 %). This is not a particularly good success rate, but the success rate varied considerably between countries and collection years (Figure 2, Figure 3).

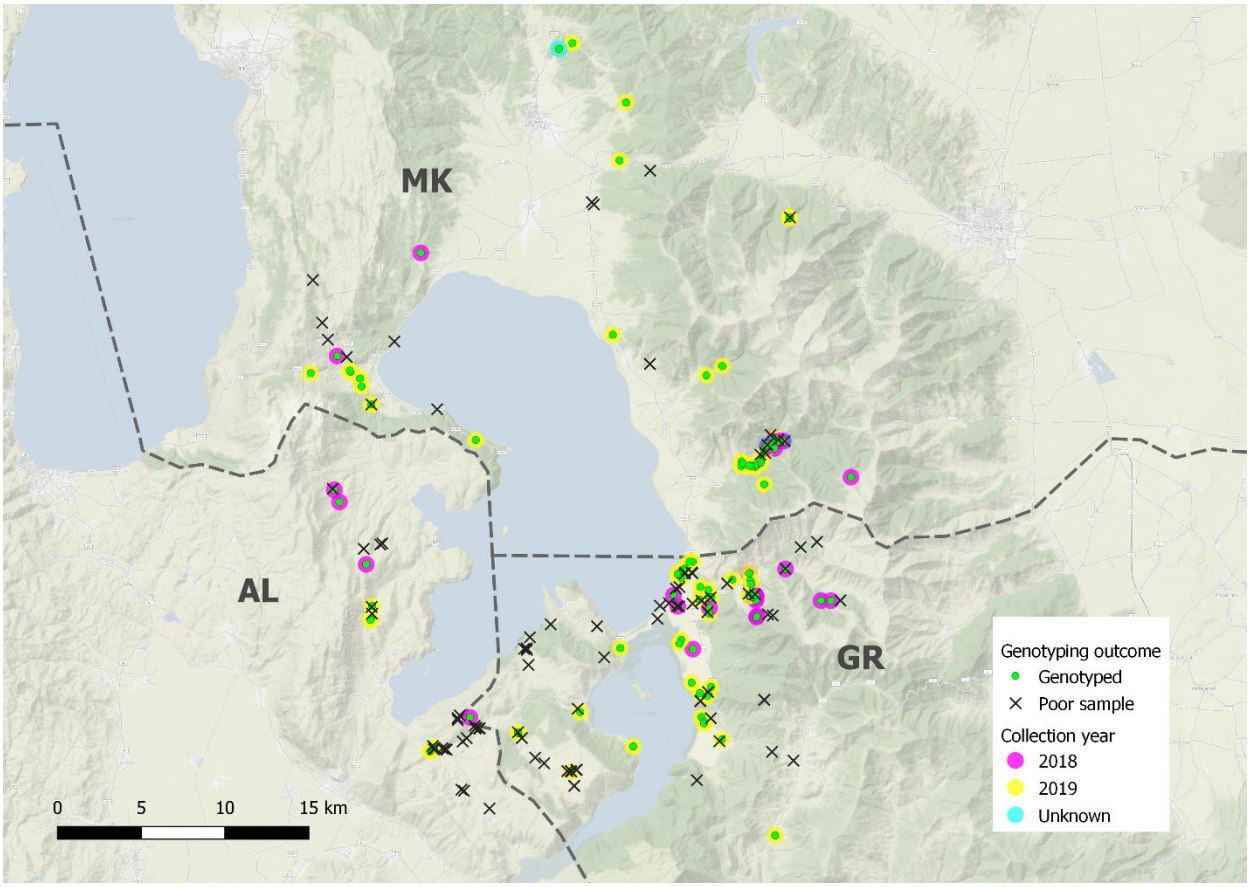

Figure 2: Geographic distribution of collected samples, genotyping success and year when a sample was collected.

In North Macedonia, we managed to genotype 49 out of 69 samples (71.0 %), which is well within expectations. The success rate was lower in Greece, where we managed to successfully genotype 59 out of 113 samples (52.2 %). This success rate is relatively low, but borderline acceptable in a study utilizing noninvasive genetic sampling. In samples from Albania, we managed to genotype just 10 out of 42 samples (23.8 %), which is extremely low.

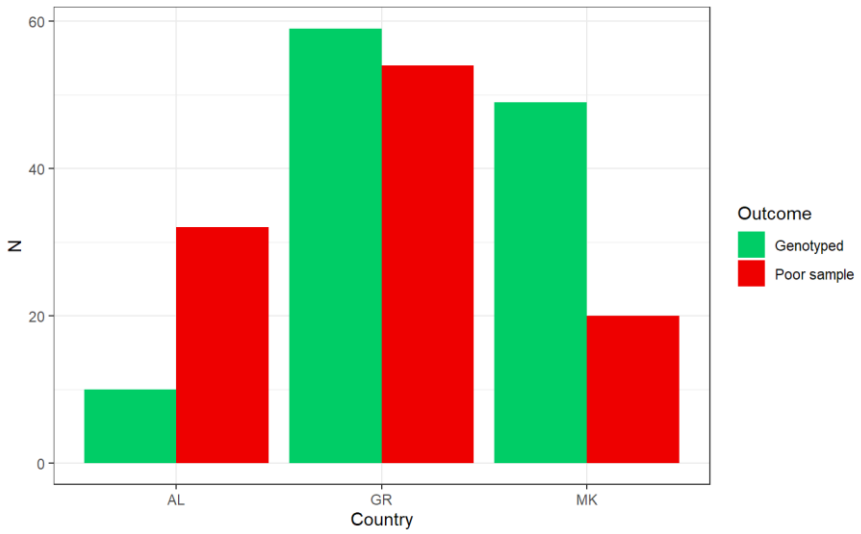

Figure 3: Genotyping success, by country.

Since samples from all countries were analyzed in a single batch, the differences in success rates can't be explained by differences in treatment during laboratory analyses.

A critical factor that influences genotyping success is the age of the scat when collected. This is estimated subjectively in the field by the person that collects a sample, and past experiences show a very high correlation between this subjectively estimated parameter and the actual genotyping success (Skrbinšek 2020). We checked if the age of scats differed between the countries (Figure 4).

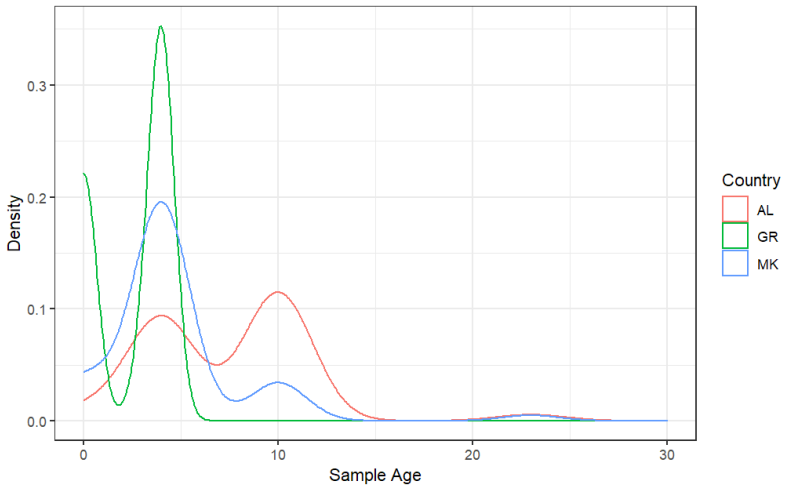

Figure 4: Age of samples, by country. Another scat from Albania, estimated to be 60 days old, is omitted from the graph.

As we see, there are considerable differences in the age of the samples, where particularly samples from Albania have been estimated as older. For Albania, most samples appeared very old when they were prepared for DNA extraction, which has been noted by the laboratory technicians and is reflected in the results.

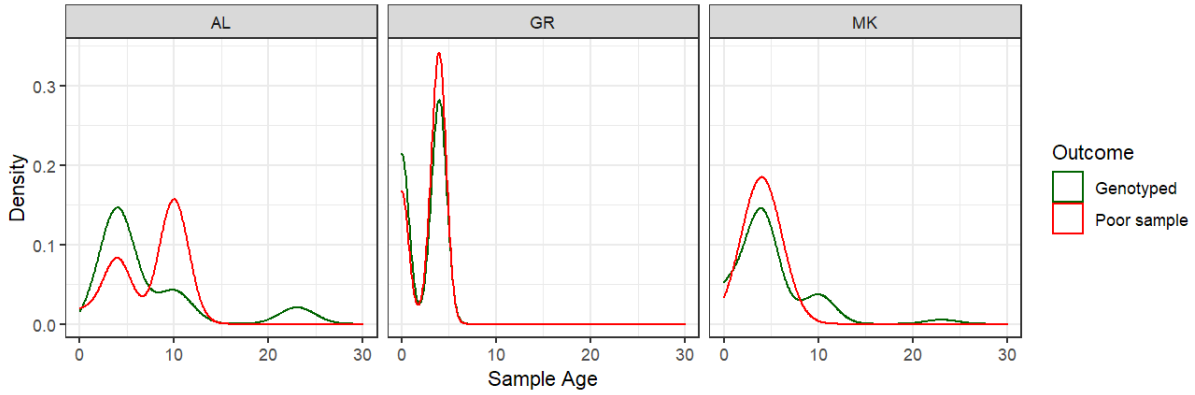

Figure 5: Genotyping outcome by country and estimated sample age.

The low genotyping success in Albania is clearly influenced by the age of the samples (Figure 5), while this connection is not obvious in Greece and North Macedonia, where samples were generally estimated to be fresher. It must be noted that the "age of scat" parameter is estimated by the person collecting the scat in the field and is as such highly subjective. However, it has proven before to be a useful predictor of genotyping success (Skrbinšek 2020).

A very interesting pattern is observed in the year the sample was collected (Table 1, Figure 6), with a much lower success rate in samples collected in 2018 compared to the samples collected in 2019.

Table 1: Genotyping success, by the year when a sample was collected.

Year	Genotyped	Poor sample	Total	% Genotyped
2018	34	56	90	37.8%
2019	76	43	119	63.9%
Unknown	8	7	15	53.3%
Total	118	106	224	47.3%

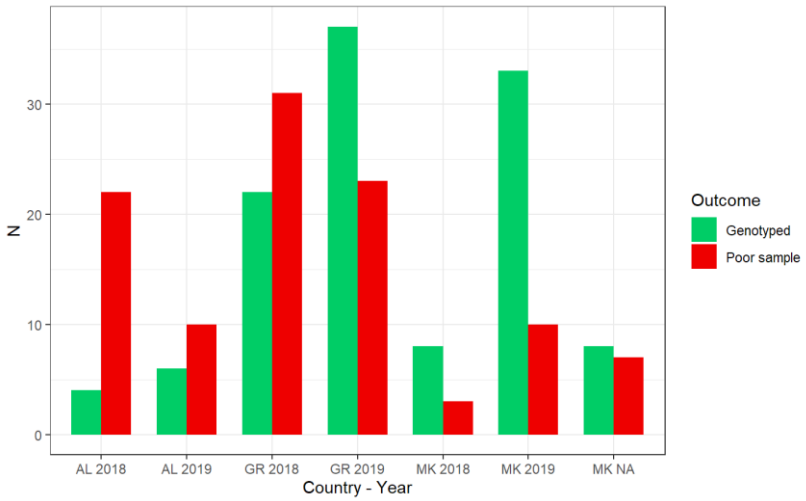

Figure 6: Genotyping success, by country and the year when a sample was collected.

An explanation for this could be the storage of the samples since samples collected in 2018 were stored for a year longer than the samples collected in 2019 before being sent to the laboratory and analyzed in 2020. The difference in genotyping success between 2018 and 2019 was lower in North Macedonia than in the other two countries (72.7 % in 2018 vs. 76.7 % in 2019), but since there were 15 samples without a recorded date from that country which had a lower success rate (53.3 %), it's difficult to draw conclusions. When testing in our laboratory, we observed no appreciable drop in the success rate in scat samples collected in ethanol over a 2-year period when they were kept at -20°C (Skrbinšek, unpublished). The samples in the present study were stored in a freezer at -20°C but were taken out of the freezer a week prior to their transportation to the laboratory, however since this was done for all samples it shouldn't result in the observed country-and-year specific effect. In any case it does seem that the storage time did have a considerable effect on the genotyping success.

An important factor that can also cause considerable problems in genotyping success is collection of samples of non-target species. With brown bears, we had issues before that field personnel, particularly volunteers with zero or little training, collected scats of other species (wild boar, horse, donkey, wolf, human, summer scats of red deer…). We noticed this in studies where we also collected full scats besides genetic samples, but in a study like the one here this could only be detected through additional (relatively expensive) laboratory analyses. However, there are considerably more samples with a very low-quality index in samples from Greece than in samples from North Macedonia (Figure 7). Quality index indicates the proportion of successful analyses in all performed analyses, for each individual locus and summarized across loci (Miquel et. al., 2006). Samples from non-target species would have the quality index zero or very close to zero (some bear loci amplify in wolves and dogs). While age of samples explains low quality index in samples from Albania, collection of samples from non-target species could explain some of the difference between genotyping success in Greece and North Macedonia (Figure 3, Figure 7).

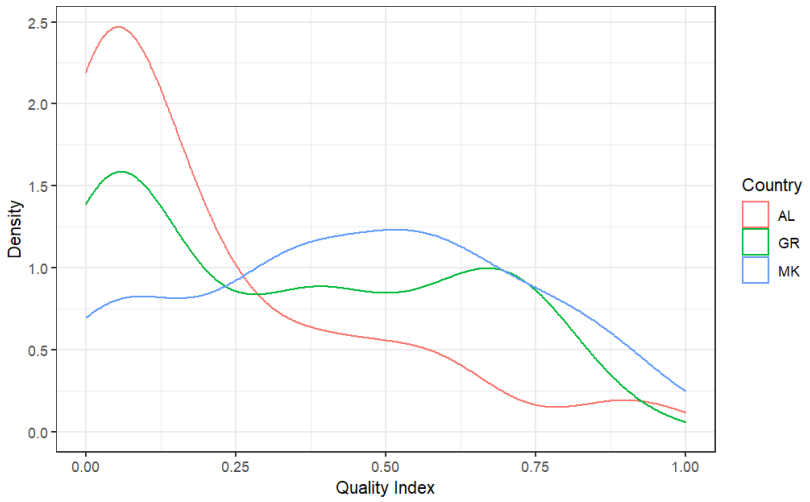

Figure 7: Density curves of samples across genotyping quality index, by country. Quality index indicates the proportion of successful analyses in all performed analyses, for each individual locus and summarized across loci.

In any case, it seems that the problem with the less-than-ideal success rate was partly in collection of old samples (Albania), partly in long (and possibly inappropriate) storage of samples before analysis. A re-evaluation of the field and sample handling protocols should be done if future studies of bears using noninvasive genetic sampling are considered in the area.

Detected animals

In the entire study we detected 51 animals, 19 females and 32 males. Distribution of samples is shown in Figure 8. Out of the 51 animals, 30 were captured (i.e., their scat was found) more than once (up to 11 times), but many recaptures were from one year to another.

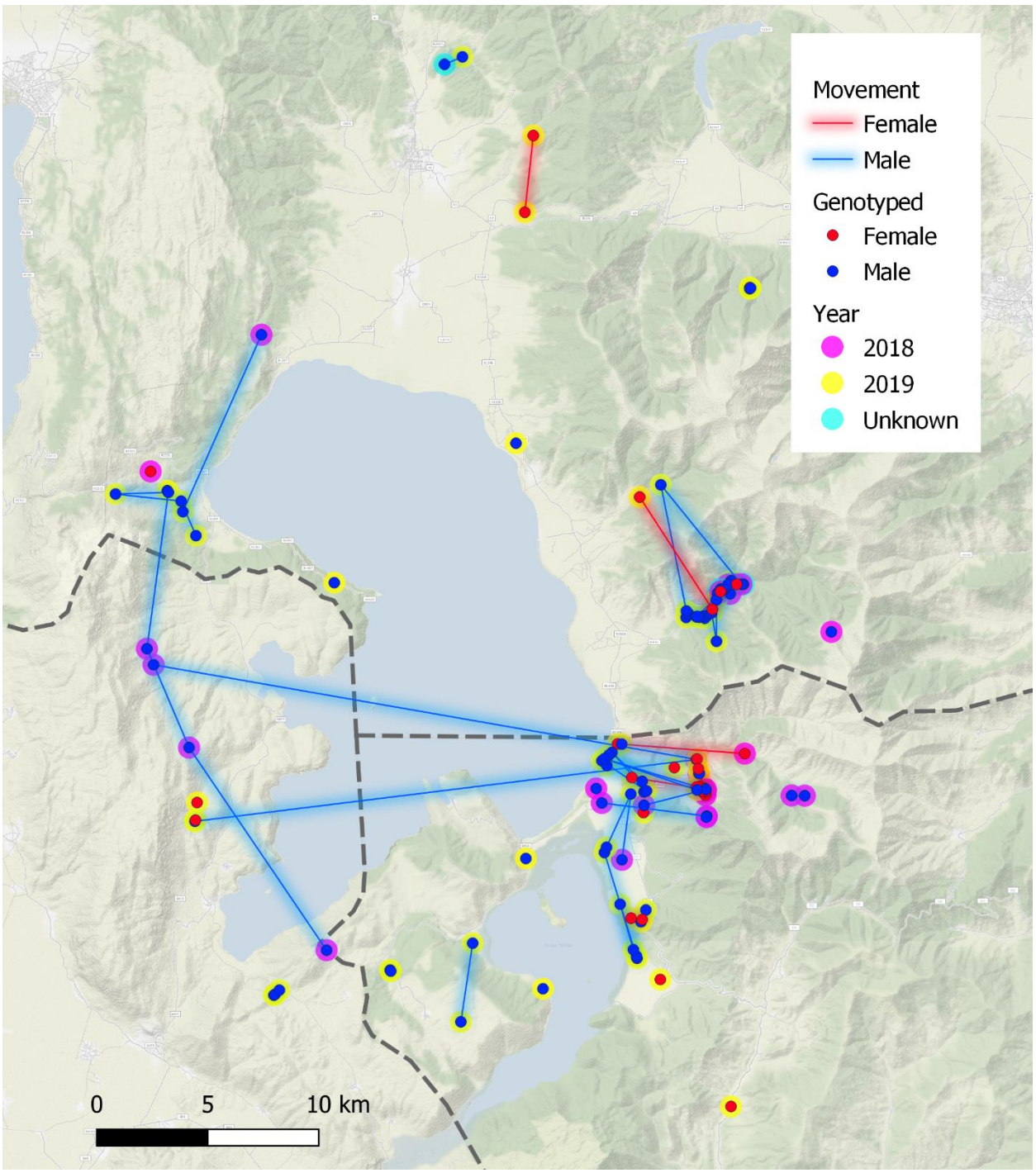

Figure 8: Samples and genotyping results. Lines connect the samples of the same animal.

There were only 8 animals (out of 21 detected) recaptured in the same year in 2018, and 9 (out of 41 detected) in 2019 (excluding 8 animals that were found each in two samples in 2019, but since both samples were collected spatially and temporally close together (within 1 km / 1 day), they should be considered a single capture). Captures / recaptures in time are shown in Figure 9.

Unfortunately, the low recapture rate in each year, when the assumption of population closure could apply, doesn't allow mark-recapture abundance estimates. Since a new cohort of young bears was born in the beginning of 2019, we'd also need considerably more samples to build a mark-recapture model using samples from both years. However, the numbers of detected animals are still useful to understand the minimum numbers of bears present in the area.

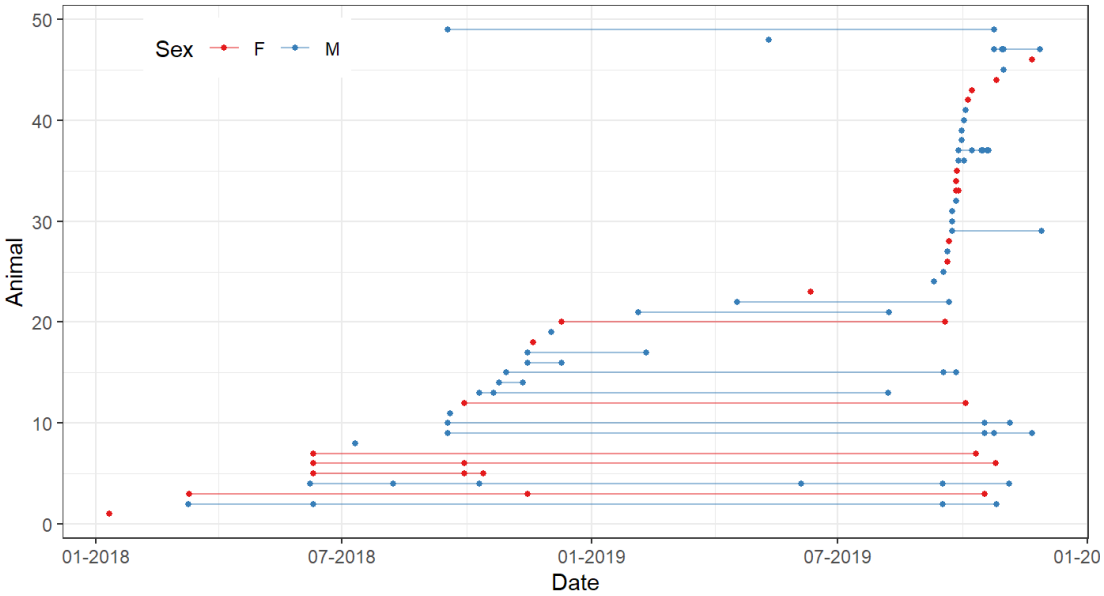

Figure 9: Captures / recaptures of animals through time. Time is on x-axis. Each animal is in its own line on y-axis, each dot is a sample, lines connect samples of the same animal.

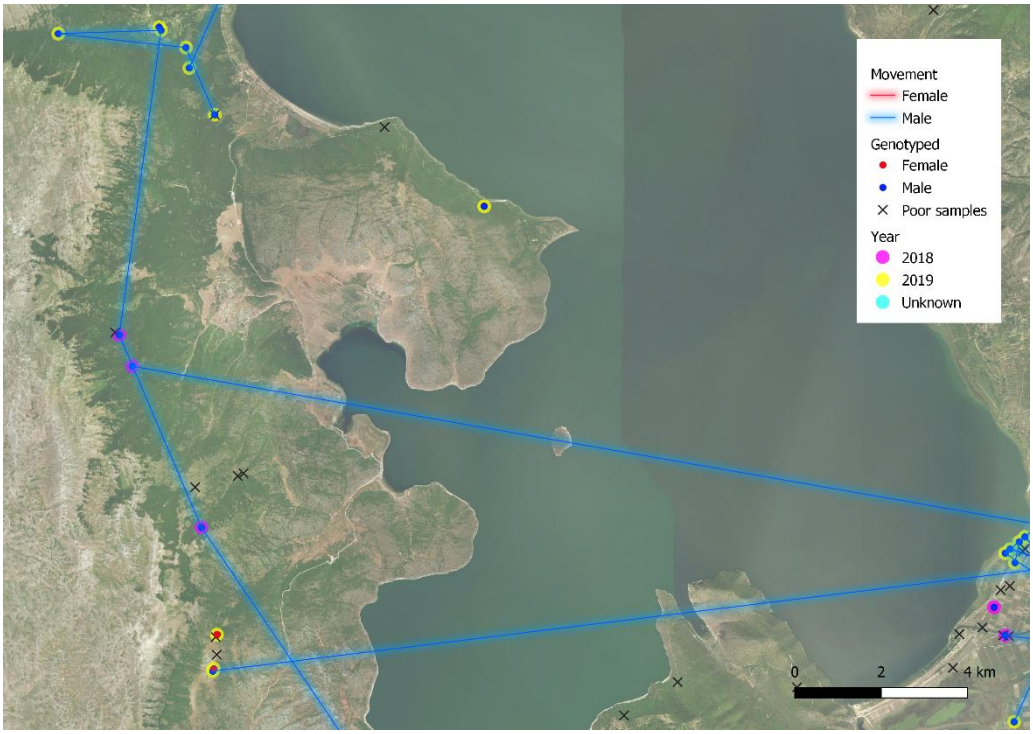

Figure 10: Samples and genotyping results, closeup of the western part of the study area. Lines connect samples of the same animal.

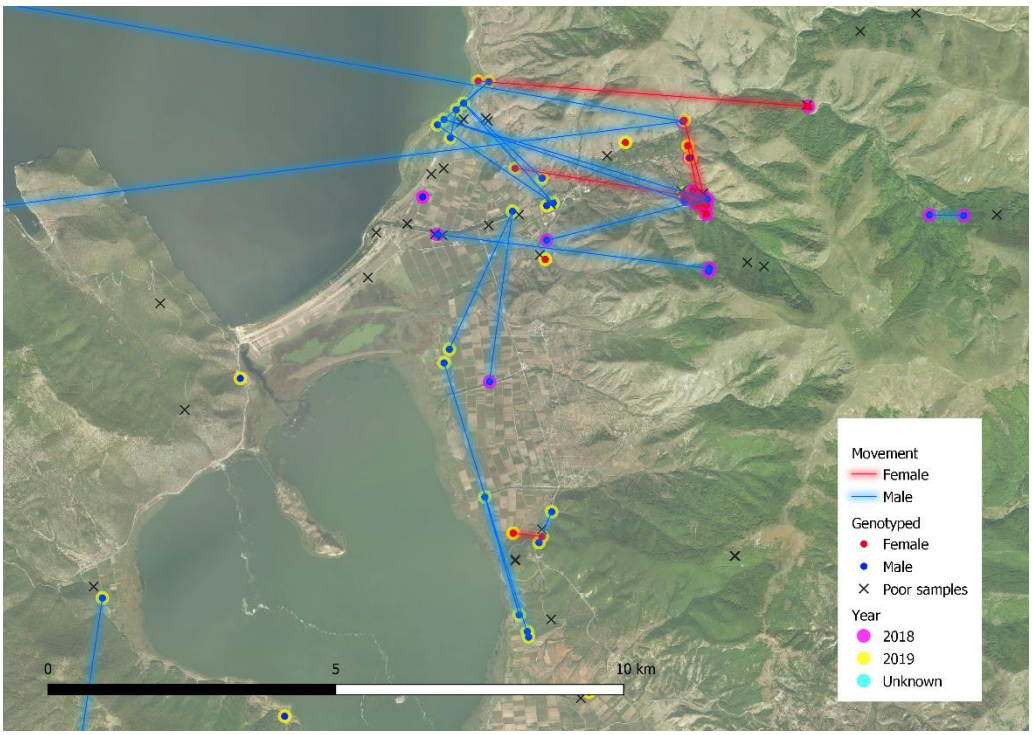

Figure 11: Samples and genotyping results, close-up of the southern part of the study area. Lines connect samples of the same animal.

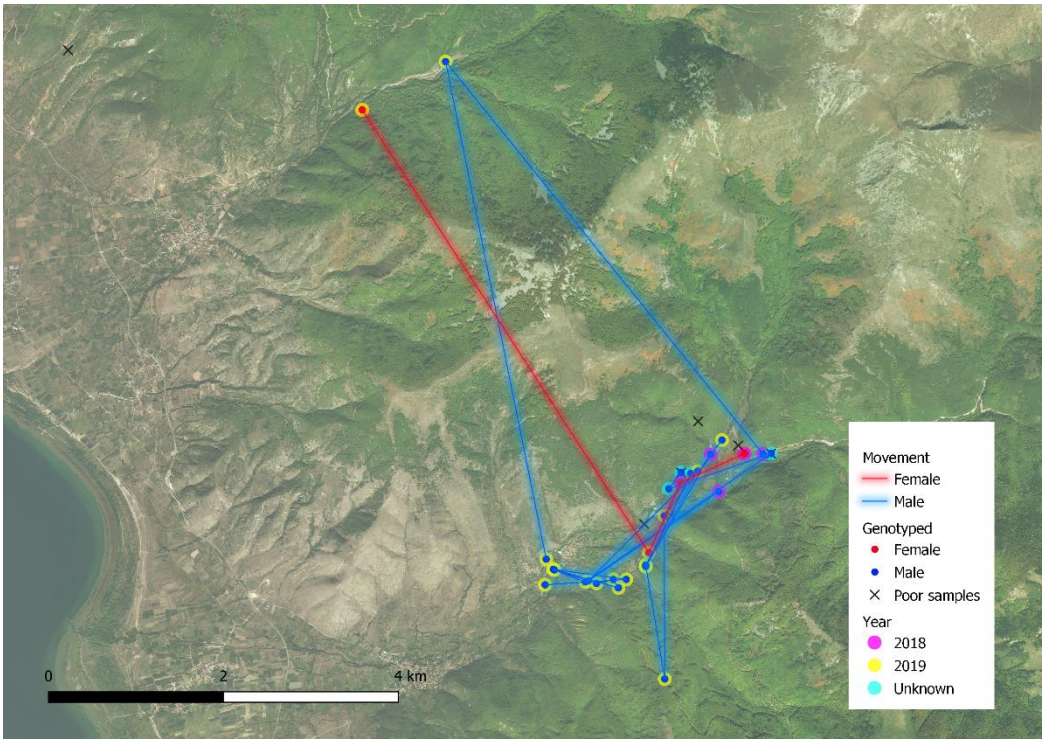

Figure 12: Samples and genotyping results, close-up of the eastern part of the study area. Lines connect samples of the same animal.

Genetic diversity

Genetic diversity in bears in the Prespa Region seems somewhat lower than that observed towards the west of the Balkans (Table 2). This fits with the findings of Karamanlidis *et al.* (2017), who found that the bears in this wider area of Greece (Vitsi – Varnoundas) had lower genetic diversity than the reference population in Slovenia. However, genetic diversity is still considerably higher than in the European bear populations that are known to be very small and endangered (Apennine and Cantabrian populations).

Table 2: Genetic diversity indices. N = number of individuals genotyped, He = expected heterozygosity, Ho = observed heterozygosity, A = allelic diversity. SE = standard error. The same markers were used in all studies.

Area	*N*	*He*	*SE He*	*Ho*	*SE Ho*	*A*	*SE A*
Prespa Region	**51**	**0.566**	**0.050**	**0.541**	**0.055**	**4.923**	**0.512**
Montenegro	53	0.649	0.020	0.630	0.021	6.077	0.560
BIH	67	0.638	0.023	0.616	0.023	6.000	0.506
Croatia - Lika E	86	0.659	0.026	0.638	0.028	5.692	0.458
Croatia - Lika W	119	0.671	0.024	0.685	0.021	5.923	0.560
Croatia - Gorski kotar	351	0.665	0.025	0.680	0.024	5.923	0.560
Slovenia	491	0.662	0.022	0.672	0.022	6.000	0.588

Discussion and conclusions

While the results of this study are not necessarily as good as we hoped, they are within the bounds that can be reasonably expected in a study that uses noninvasive genetic samples, and perfectly acceptable for a pilot stuy. However, many of the issues that decreased the study success have been identified and being understood, they can be easily corrected if similar studies are considered in the future. We consider this an important outcome.

Genotyping success

When noninvasive sampling is done in a certain area and by a certain organization for the first time, it often meets considerable logistic problems, and this study was no exception. There are many details that need to be considered – from broad ones like how the sampling will be organized, to some very specific like selection of a tube for samples and conditions for sample storage – that all contribute to the study's ultimate success, or lack of it. Some of the problems can be solved if an expert with considerable experience in noninvasive genetic sampling is involved from the conception of the study onwards, but even so there may be conditions specific to the area or the project that may impact the outcomes of the study in an unforeseen manner. This is what makes pilot studies extremely important, and how the study described here should be considered.

The success rate in the study was not particularly good, despite considerable laboratory efforts. In our previous studies we analyzed a sample that passed initial screening (indicating that it may have some viable bear DNA) up to 8 times – if we couldn't get a reliable genotype, the sample was discarded. Because of the relatively low DNA quality in this study and many "marginal", barely amplifying samples, we increased this to 16, meaning that each sample was analyzed up to 16 times before being discarded. This helped us salvage some results but would be ultimately too expensive in a larger study.

What is important though is that we managed to identify the problems, and all are preventable if another large study is attempted.

In samples from Albania, the main problem was that old samples were collected, driving the success rate to extremely low. There was also a problem that inappropriate, leaking tubes were used, which besides creating a mess also probably had a negative effect on conservation of DNA in the sample where ethanol leaked out and the sample was left somewhat dry. This problem was present also for samples from North Macedonia, but to a lesser degree.

In samples from Greece, we're guessing that there may be a problem with collection of non-target species, or general collection procedures (e.g., collection of too much scat with not enough ethanol to conserve it). While we cannot support this without additional (expensive) labwork, there are more samples of very low quality from Greece than from North Macedonia, and there are several people that collected large numbers of samples that had low success rate (data not shown here). We encountered this in previous studies with volunteers and attributed the problems to insufficient training and/or poor

instructions for sample collection.

Samples from North Macedonia, on the other hand, performed better, but 18 samples didn't have the collection date recorded, and 4 samples didn't have coordinates. Samples that lack field data are of very limited use, or even useless for many of typical downstream analyses.

Lastly, the critical problem for genotyping success seems to have been sample storage. There is a considerable decrease in genotyping success in samples collected in 2018 compared to the samples collected in 2019, and both were sent to the laboratory in spring/summer 2020. This drop in genotyping success suggests problems with sample storage that severely affected 2018 samples, but possibly had also an effect on samples from 2019.

While the combination of these effects contributed to the relatively low success of the study, the fact that we were able to identify them allows for considerable optimism if a similar or larger study would be repeated. All of the mentioned problems are relatively simple to address, indicating that a highly successful study could be done in the area in the future.

Bear numbers, sex ratio and spatial distribution

We detected 51 animals altogether, 19 females and 32 males. This indicates a considerable skew towards males, which probably doesn't reflect the real sex ratio in the population but is probably caused by sampling design and implementation. In studies where the study area is relatively small compared to the species homerange, differential space use between sexes can cause shifts in observed sex ratios. Since male bears often have considerably larger homeranges than females, even with an equal sex ratio in the population we would expect that more male bears would have a part of their homerange within the sampling area and could possibly be sampled. Another source of the bias could be nonrandom sampling if areas that are preferentially visited by male bears have been sampled more intensively. A discussion about that should be started with the people that are more familiar with the field sampling design.

Unfortunately, the data don't allow for a mark-recapture estimate of bear abundance in the area. Designing a mark-recapture study for a relatively small area is difficult as the edge effect of animals leaving the area and entering will be very strong, causing capture heterogeneity between the animals. While in principle doable, in such a study sampling should be very intensive and short (our experiences indicate that 2-3 months would possibly be ok, the shorter, the better) so that most animals would stay in the area and that recapture rates would be high. It also helps if the sampling is done in autumn/early winter since bears are in their hyperphagia period at the time and usually move around less.

While there is not enough data to make strong conclusions about spatial patterns, we can see that many bears remain in approximately the same area from one year to the other, and that some bears cross the borders between countries and can be found in different parts of the sampling area. There were two bears observed that made longer excursions, both males.

Genetic diversity

While not the primary goal of this study, the genetic diversity parameters observed were in line with the findings of previous studies done in the area and show a relatively genetically diverse population, but with genetic diversity that is lower than in the western part of the Dinaric – Pindos population.

Recommendations

Being the first study of brown bears in the area that used noninvasive genetic sampling, the study provided important insights. We will summarize the critical issues we encountered and suggest recommendations for future studies. We believe that by using the experiences gained here, we can make future genetic studies of brown bears in this area very efficient and successful.

Increasing genotyping success

While the total genotyping success rate in the study was not as high as we hoped, it is still within reasonable bounds for a study utilizing noninvasive genetic samples. However, one extremely important result of the study is that the problems were identified and can be corrected in future studies.

The most critical, and probably the easiest to solve, is the problem with sample storage. We recommend that samples are kept in a cool and dark place as much as possible immediately when they are collected in the field and transferred to a freezer (-20 °C) as soon as possible. They should also be sent to the laboratory for analysis as soon as practical. A correct ratio of ethanol and scat (at least 4:1, but higher ratios are better) should be observed during collection to allow for good conservation of the DNA.

There are two other critical issues that have been identified or indicated: sample age and (presumably) correct identification bear scats. Both can be easily solved by providing detailed, written instructions, and if possible, by organizing short training meetings for anyone involved in sampling. This can considerably increase genotyping success and provide much more useful data for given effort and costs.

A critical detail that must be mentioned, particularly if sampling is done with volunteers, are good sampling tubes that have been tested not to leak even in extreme circumstances. This is not difficult to do – we usually do three tests: we fill a tube with ethanol and 1) someone heavy jumps on it, preferably several times, 2) season permitting, we leave it under a car windshield on sun in summer, and 3) someone strong tries to tighten the cap with bare hands as strong as possible, trying to break the cap. If the tube survives these tests, it is suitable to be used in the field. A poor tube with collected scat leaking all over a backpack/bag while in the field can really ruin a person’s day, and it's questionable if such a volunteer would still collect samples in the future.

Sampling design

There are several recommendations for study design considerations that should be taken into account if future similar studies in the area are planned:

- The sampling area should be increased, if possible. The sampling area seems relatively small compared to a brown bear home range, particularly for males. This causes a pronounced edge

effect, creates capture heterogeneity and makes any abundance estimates with mark-recapture difficult.

- Sampling fieldwork should be planned so that most of the study area is systematically covered, to have the capture probability between animals (at least of the same sex) as equal as possible. Preferential sampling at sites more attractive to some animals (e.g., males) should be avoided – such sites should be surveyed, but samples from them should not represent the majority of samples.
- If possible, sampling effort should be recorded to enable population density estimates using spatially explicit capture recapture methods.
- To estimate abundance, sampling should be highly intensive and limited to a single reproductive season to avoid serious violations of the population closure assumption. If the goal is estimating population dynamics, sampling should be done in at least three consecutive years to enable the "robust design" sampling scheme and usage of corresponding mark-recapture models.
- Mark-recapture simulations should be used to facilitate correct scaling of the sampling effort..

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