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Report on analysis of genetic samples collected in 2017 – 2018 on brown bears (*Ursus arctos*), Eurasian lynx (*Lynx lynx*) and grey wolf (*Canis lupus*) in a pilot area in Southern Carpathians, Romania

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1 INTRODUCTION

The goal of the study was to analyze noninvasive genetic samples of brown bear, lynx and grey wolves from a pilot area in Southern Carpathians, Romania. The study was conducted in autumn 2017 for brown bear and in winter 2017-2018 for wolf and lynx and was designed as a start of a long-term genetic monitoring of these species in the area.

While the study suffered from some minor problems that are common when endeavors like this are started, we can clearly call it a success. Results for bears and wolves are very good. Results for lynx are more modest, but then again lynx is an extremely difficult species for genetic monitoring. All in all, we feel that the study provides a solid foundation for long-term monitoring, while the experiences gained are sure to make the results even better in the years to come.

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METHODS

2.1 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.

2.2 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. We used a liquid handling robot (Hamilton Starlet) to achieve reliable, error-free and fast DNA extraction. Besides speeding the analyses, use of the liquid handling robot practically eliminated the possibility of a sample mixup since all sample handling is done automatically, and sample IDs read and handled through barcodes. The liquid handling robot is located in the "noninvasive genetics laboratory" and used exclusively for noninvasive and historic samples.

Since their number is manageable, DNA extraction from tissue samples is done using manual DNA extraction kit (Sigma GenElute) in the "tissue laboratory".

2.3 GENOTYPING

Genotyping methods differ by the species, as species-specific genetic markers are used.

2.3.1 Brown bear

We used a new method described by De Barba et al. (2016) for genotyping. The method taps the power of next generation (high-throughput) sequencing (NGS), solves 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. (2016) 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. 12 or 13 libraries are analyzed simultaneously in a single HiSeq run.

Once the sequences are received (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 rapidly 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.

2.3.2 Wolf – basic genotyping

Samples were genotyped at 16 canine unlinked autosomal microsatellite loci in one PCR multiplex (AHT137, AHTh171, AHTh260, AHTk211, AHTk253, CXX279, FH2054, FH2848, INRA21, INU030, INU055, REN162C04, REN169D01, REN169O18, REN247M23, REN54P11) and the Amelogenin locus, which was used for sex determination. Prior testing showed that this marker set does not give specific PCR products for domestic or wild ungulate DNA (unpublished data).

In the first screening process, each sample was amplified with the full genotyping PCR protocol twice and analyzed on an automatic sequencer (Applied Biosystem ABI 3130xl Genetic Analyzer). Samples that provided no specific PCR products at that stage were discarded; the other were genotyped up to eight times, with reliability of the genotype being checked with Reliotype (Miller, Joyce, & Waits, 2002) maximum-likelihood approach after each genotyping run.

2.3.3 Wolf - extended genotyping for "reference" samples

The best-amplifying ("reference") sample of each individual animal was amplified using a panel of additional 10 microsatellite loci (C09_250, C20_253, CPH12, CPH5, CPH7, CPH8, CPH9, Cxx_121, FH2010, FH2145) for parentage and hybridization analysis, bringing the total number of useable microsatellite markers to 26. Another sex-ID locus (SRY) was used to double-check designation of sex. The same quality-assurance procedure that was applied for the individual-ID panel was also applied for the extended panel.

2.3.4 Lynx

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

All PCRs were done using Qiagen Multiplex PCR kit. We prepared 10 μL reactions – 5 μL of Qiagen Mastermix, 1 μL of Q solution, 2 μL of template DNA and 2 μL of primer mix and water to obtain the appropriate concentration. The reactions were first denatured at 95°C for 15 minutes, then cycled for 50 cycles at 94°C for 40 seconds, 60°C for 90 seconds and 72°C for 90 seconds. We followed this with a final extension step of 30 minutes at 60°C. The other procedures were the same as with the wolf samples.

2.4 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 strictest quality assurance protocols, are unavoidable in noninvasive samples (Taberlet, Waits, & Luikart, 1999; Lisette P Waits & Paetkau, 2005). Incorrect matching can cause considerable biases in mark-recapture estimates (Roon, Thomas, Kendall, & Waits, 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 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 (L P 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 using tissue samples with a very low error rate (T. 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.

2.5 MARK-RECAPTURE ANALYSIS (BEAR AND WOLF)

The minimum number of animals (directly detected as the number of different genotypes), while useful, is rarely enough for management purposes. The critical information that is usually needed is the number of animals that we didn't detect during the sampling, and hence the total number of animals in the sampled population. This problem is typically tackled through mark recapture modelling.

However, one needs appropriate quality data to use these powerful techniques. The first requirement is that there is enough data – without enough recaptures there is no mark-recapture model. The second requirement is for the data to reasonably fit model assumptions. In models designed for abundance estimates this usually means that the population must be demographically closed (no immigration/emigration, no births and no (undetected) deaths during sampling). Another important assumption is that each animal has the same probability of being "captured" (in our case this means having its sample collected and successfully genotyped). While these assumptions are violated to a degree in each empirical study, the task of the researcher is to limit these violations as much as reasonably possible to obtain a valid result.

We used several mark-recapture modelling approaches, as much as data for each species allowed. 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 is very robust in estimating the lower bound on abundance. We also tested the Darroch Mh (Mt) model. The last two models were fitted using the R package RCapture (Baillargeon & Rivest, 2007)).

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). Darroch's model is also one of the "classic" models, usually parametrized to capture heterogeneity that's been developed through time. Although we are not aware of strict simulation tests of its performance, we're including it here as a reference since it was often selected as the superior model in Rcapture model selection (using AIC or BIC).

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 designed to be relatively short and before reproduction, during the autumn hyperphagia period, we assumed that the sampled population should behave as demographically closed. We used the Pradel model with recruitment and survival parametrization in MARK to check this assumption.

The sampling area is demographically open without providing any significant physical obstacles to bear movement. This means that the issue of edge effect must be taken into consideration. This means that the actual mark-recapture estimate is for a "superpopulation" of animals that may have a part or much of their homerange outside of the study area but wander into the study area enough that they can be sampled. We used the correction proposed by (Wilson & Anderson, 1985) to correct for the edge effect and estimate of the "moment" population size. We used detected pairwise distances between locations of samples of the same animal to calculate W, the width of the strip outside of the study area where the animals would have a non-negligible probability of being included in sampling. Because of expected differences in habitat use, W was calculated separately for each sex. To obtain the moment population size estimate, we used the As/At as the correction factor for our superpopulation estimate, where As is the size of the study area, and At the total area including the edge strip.

2.6 HYBRIDIZATION WITH DOMESTIC DOGS (WOLF)

Hybridization with dogs is an important threat to wolf conservation in many areas (Godinho et al., 2011). We used genotypes of 47 domestic dogs and 176 reference wolves from Dinaric Mountains as reference samples to determine if the detected canids are pure wolves. We used Bayesian clustering in program Structure (Pritchard, Stephens, & Donnelly, 2000) to detect hybrids. We preformed simulations in program HybridLab (Nielsen, Bach, & Kotlicki, 2006) to simulate wolf-dog hybridization, and used these simulated hybrids to determine hybridization thresholds.

2.7 PARENTAGE AND SIBSHIP ASSIGNMENTS

Parentage and sibship assignments enable us to identify family groups and estimate the number of packs present in the study area even when the data is too sparse to allow for a reliable mark-recapture estimate. We used program Colony (Jones & Wang, 2010) to simultaneously assign parentage and sibship assignment and determine family groups (packs) in the area. The Colony method is particularly powerful since it enables both parentage in sibship assignments in the same model, providing more efficient use of available data.

2.8 DATA ANALYSIS

Genetic data were prepared in laboratory database (MisBase), which we use also to keep the record of the field data. The data were exported into GIS software (QGIS) to determine spatial characteristics of each data point (inside/outside of the samping area locations).

All non-GIS analyses were run in R (R Development Core Team 2018), with the exception of the abovementioned parentage analysis and mark-recapture analysis in program MARK. We programmed a number of functions to analyze and visualize genotyping success data, parentage results and mark-recapture data and results.

3 RESULTS AND DISCUSSION – BROWN BEAR

3.1 LABORATORY ANALYSIS AND GENOTYPING SUCCESS.

In total, we analyzed 780 samples: 536 samples of feces, 238 hair samples, 5 tissue samples and 1 urine sample. Genotyping success is summarized in Table 1.

Table 1: Genotyping success, all samples.

Outcome	N	%
Successfully Genotyped	514	65.9 %
Mixed Sample	22	2.8 %
Poor Sample	244	31.3 %

Success rates differed by sample type (Table 2). Collected scats were relatively old (2.6 days estimated mean age), which contributed to the lower success rates (Figure 1). Success rate drops rapidly as scat ages, from over 90% for scats estimated to be 0 days old to around 40% in scats estimated to be 4-5 days old. In a similar study with nearly identical methodology in Slovenia and Croatia, we had 79% success rate in Slovenia where average estimated sample age was 1.48 days, and 66% success rate in Croatia where the average sample age was 2.08 days. In light of that, results seem expected and acceptable.

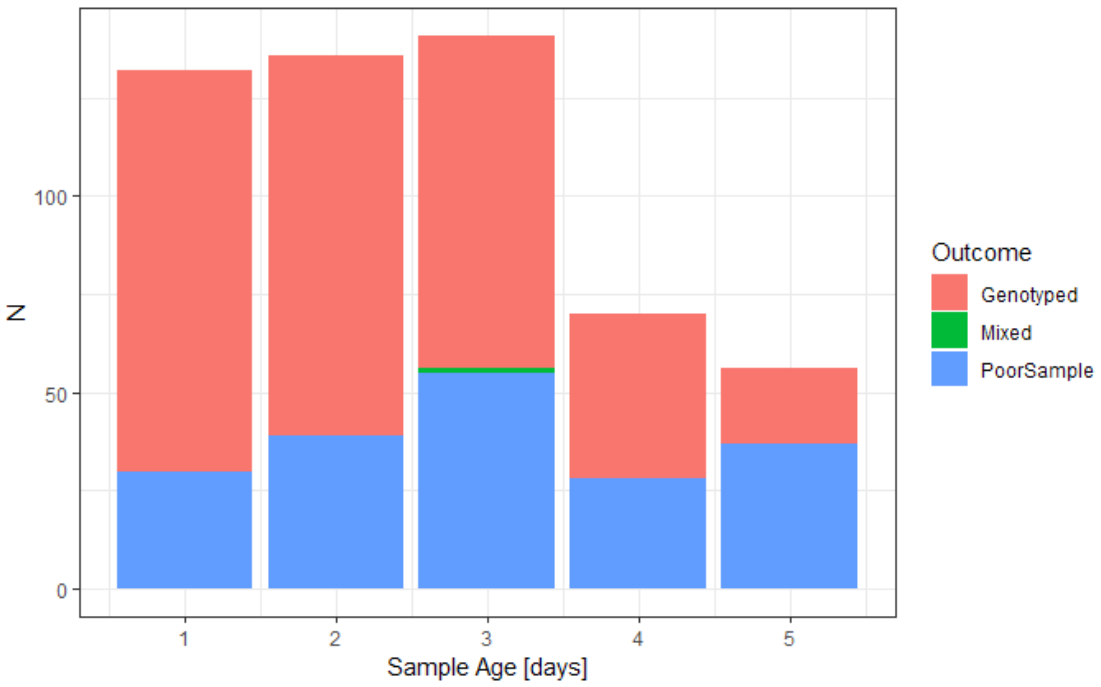

Figure 1: Genotyping success of scat samples by subjectively estimated age of the scat.

Table 2: Genotyping success, by sample type.

S. Type	Genotyped	Mixed	PoorSample	Total	% Genotyped	% Mixed	% Poor
Feces	346	1	189	536	64.6	0.1%	35.3%
Hair	167	21	50	238	70.2%	8.8%	21.0%
Tissue			5				
Urine	1						

Although the success rate from hair samples was good, there was a considerable number of mixed samples (genotypes of different individuals in the same sample). Such samples were discarded.

3.2 SPATIAL AND TEMPORAL DISTRIBUTION OF SAMPLES AND DETECTED INDIVIDUALS

While most samples were collected during the 2017 autumn sampling season, some samples were collected earlier or later. These were removed from the mark-recapture analysis (Figure 2).

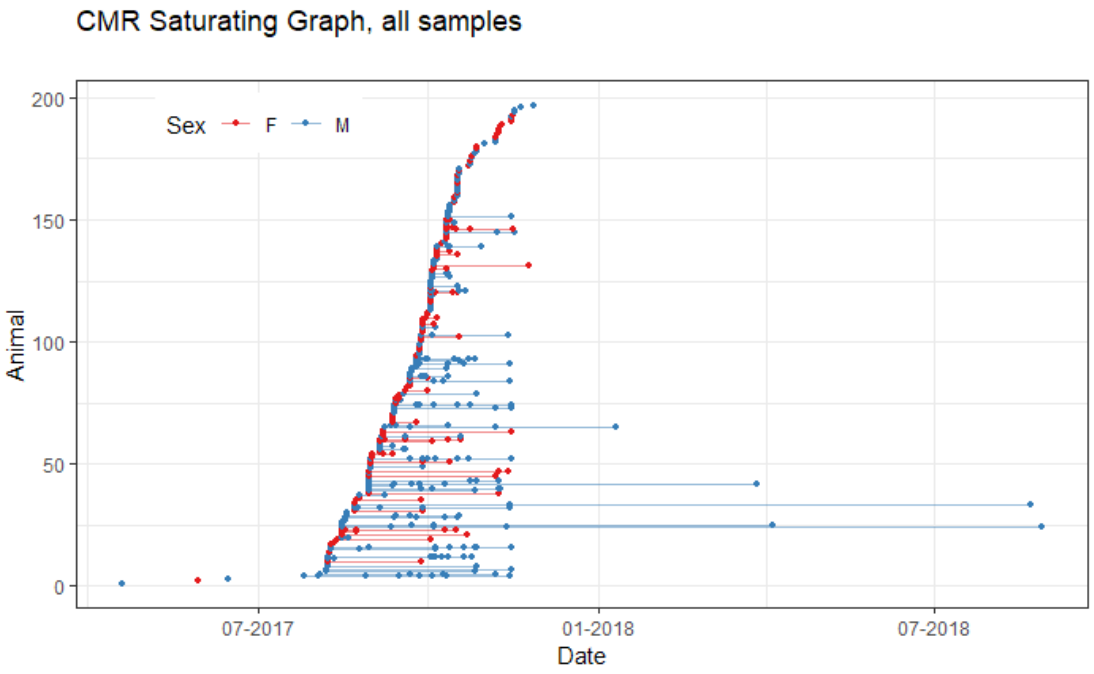

Figure 2: Mark-recapture saturating graph. Each dot is a sample, each line connects samples of the same animal.

Similarly, while most samples were collected within the sampling area, some were collected outside. These were also removed from downstream analyses (Figure 3).

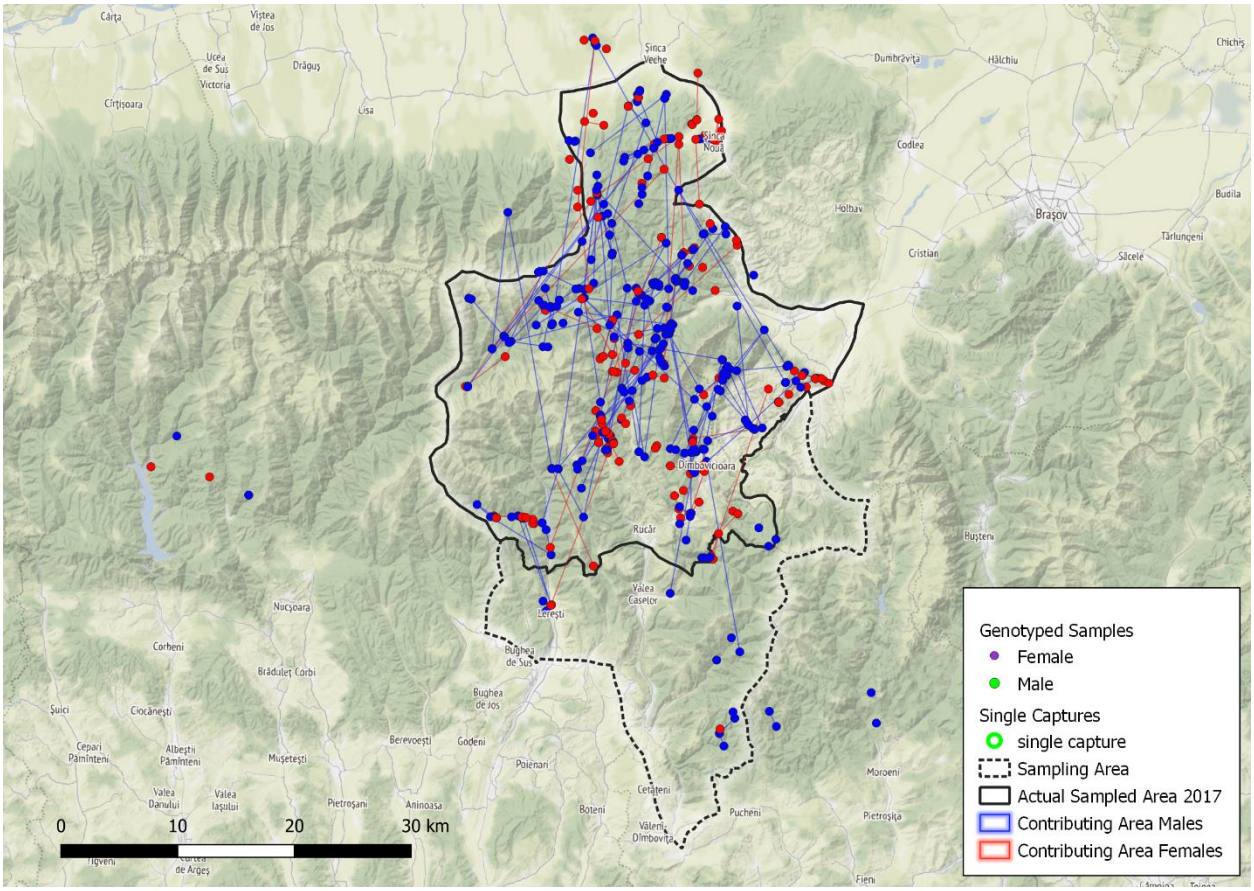

Figure 3: Genotyped samples and sampling area. Samples of the same animal are collected by line in the order they were collected. The actual sampled area in 2017 was smaller, and the samples outside were discarded for the downstream analysis.

The mark-recapture saturating graph looks much better (Figure 4, top). However, the sampling for females looks much scarcer, with less total samples and less recaptures (Figure 4, below).

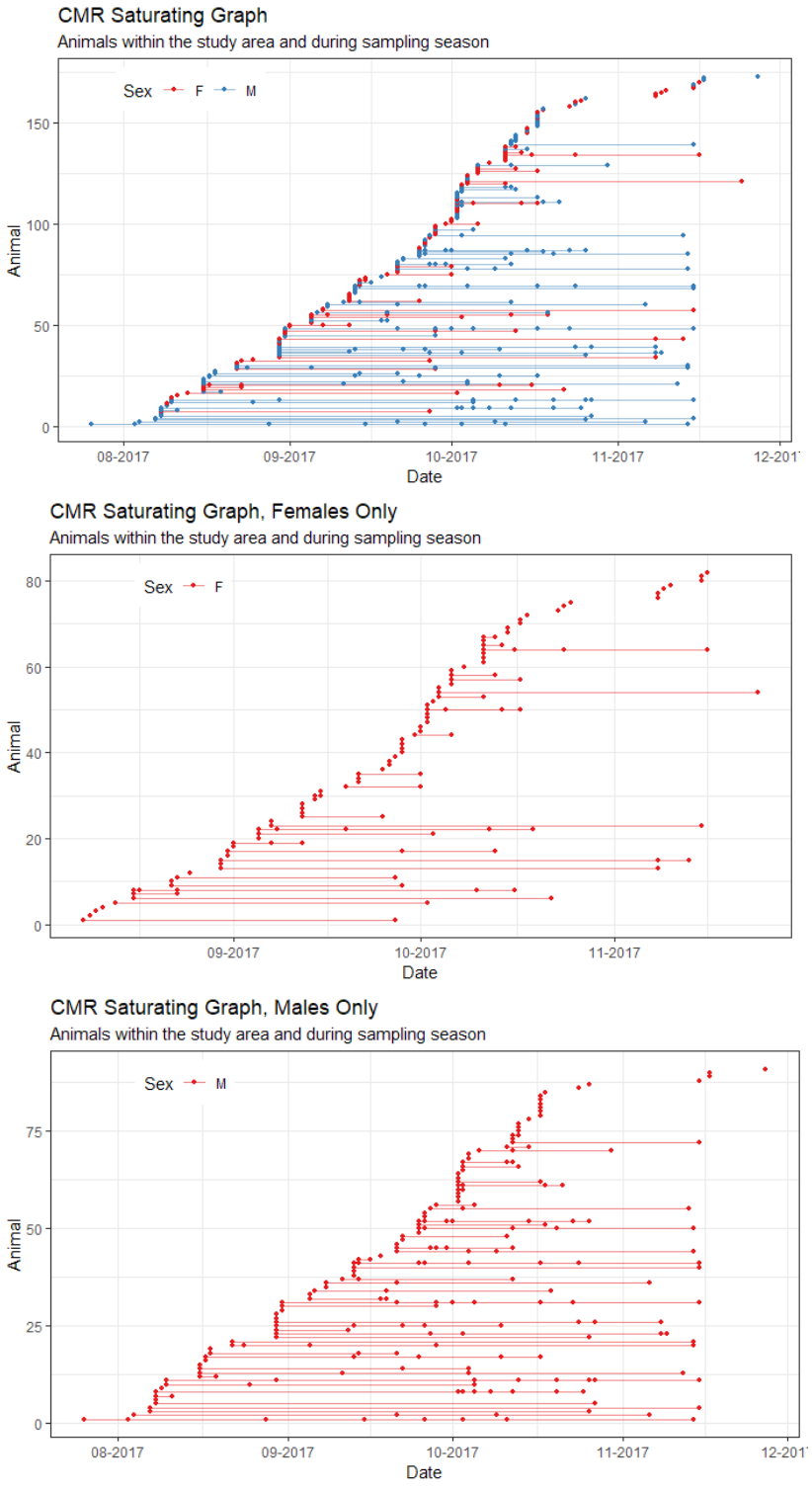

Figure 4: Mark-recapture saturating graph – all animals and each sex separately. Each dot is a sample, each line connects samples of the same animal.

3.3 DETECTED ANIMALS AND SEX BIAS IN SAMPLING

There were more males detected than females (Table 3), but not significantly more considering that there were almost twice as many male samples collected than female samples. However, it seems that there was a higher probability to detect males than to detect females in this sampling. This bias is interesting, and we haven't detected it in previous studies elsewhere. The main issue seems to be hair sample from rubbing trees (Table 3), and males seem to have a much higher preference for rubbing trees than females. Interestingly, there also seems to be a bias in detection of different sexes in scat samples, but this may be an artifact of the edge effect (see mark-recapture results below).

Table 3: Number of detected animals within the 2017 sampling area by sex and numbers of samples of each sex by sample type.

Detected animals by sex				
GeneticSex	N			
F	83			
M	91			
Total	173			
Genotyped samples, by sample type and sex				
Sample Type	Females	Males	Total	% Females
Feces	131	174	305	42.9%
Hair	31	121	152	20.4%
Total	162	295	457	35.45%

3.4 MARK-RECAPTURE RESULTS

A number of modelling approaches were tested (see the "methods" section). A common finding was that regardless of the approach, the model selection procedures (where available) preferred the models that relaxed the capture homogeneity assumption (heterogeneity models), indicating some capture heterogeneity in the data.

3.4.1 MARK analysis & closure test

For analysis in MARK we organized the samples into sampling intervals, taking care that each detected animal would have between 15% and 30% probability of being detected within a certain interval. At the upper and lower part of this range, we ended up with 8 or 6 intervals, respectively. Both datasets were analyzed, but the 6-interval data seemed to have better properties (denser data, higher capture probability in each interval, less heterogeneity) and its results are being reported. Goodness-of-fit test with the median c-hat method showed good fit of the model (estimated c-hat = 1.038, ideal fit is c-hat = 1).

MARK has a very well-defined model selection procedure that allows for hypothesis testing using an information-theoretic approach. This makes it useful for testing various assumptions about the dataset, and the results can be used to better formulate and interpret other modelling approaches.

Population closure test with the Pradel model (with recruitment and survival parameterization) supported the assumption of population closure since model that fixed survival at 1 and recruitment at 0 was selected as the best model, and was considerably better than the model where these two parameters were estimated from the data (dAIC = 3.99). Estimated from the data, survival for both sexes was estimated as >0.99, and recruitment <0.01, further supporting the closure assumption.

We generated an a-priori set of 11 plausible models using the Huggins modelling approach. The Selected models included capture heterogeneity and sex (modelling capture probability differently for males and females). Considering the high sampling bias, particularly in hair traps, this makes sense.

3.4.2 Capwire and Rcapture analysis

Both approaches use continuous sampling data, which fits better to how the samples were really collected in the field. Because of this, we could expect these models to have narrower confidence intervals than MARK models (they should use the data more efficiently). However, we removed autocorrelated samples from the analysis. If two or more samples of the same animal were found on the same day less than 500 meters apart, they were considered statistically non-independent and only one of these samples was retained for the analysis.

The Capwire model selection preferred the model that included heterogeneity of capture (TIRM). Since both the direct genotyping results and MARK analysis indicated different capture probability for each sex, and Capwire doesn't allow including groups directly in the model, we ran the models for each sex separately and added both results to obtain the total number of animals.

We took the similar approach with the Rcapture analysis. Since aggregation into sampling intervals provides benefits by decreasing capture heterogeneity, we also ran the Mh-Chao model in MARK. The results are presented in Table 4 and Figure 5. All modelling approaches provided similar results.

Table 4: Results of superpopulation size estimates of brown bears obtained through different mark-recapture modelling approaches. The "sex = All" models were fitted with all data regardless of sex, and "sex = Sum M+F" models for males and females were fitted separately and the results summed. In MARK models the sex information was fitted explicitly in the model, but the results were reported for each sex separately and summed. The models in ***bold print*** *and denoted by * have been considered the best and used in downstream analysis.*

model	sex	abundance	cid	ciu
Capwire ECM	All	201	187	215
Capwire ECM	Females	120	100	145
Capwire ECM	Males	98	91	103
Capwire ECM	Sum M+F	218	191	248
Capwire PART	All	308	291	381
Capwire PART	Females	174	164	206
Capwire PART	Males	133	124	171
Capwire PART	Sum M+F	307	288	377
Capwire TIRM	All	270	262	341
Capwire TIRM*	**Females**	**162**	**145**	**200**
Capwire TIRM*	**Males**	**128**	**113**	**145**
Capwire TIRM*	**Sum M+F**	**290**	**258**	**345**
Darroch	All	269	230	309
Darroch	Females	176	105	246
Darroch	Males	122	103	141
Darroch	Sum M+F	298	208	387
MhChao	All	255	209	301
MhChao	Females	141	106	177
MhChao	Males	125	96	154
MhChao	Sum M+F	266	202	331
MARK Mh*g 6int	All			
MARK Mh*g 6int	Females	169	115	311
MARK Mh*g 6int	Males	136	112	187
MARK Mh*g 6int	Sum M+F	305	227	498
MhChao MARK	All			
MhChao MARK	Females	171	127	258
MhChao MARK	Males	141	116	194
MhChao MARK	Sum M+F	312	243	452

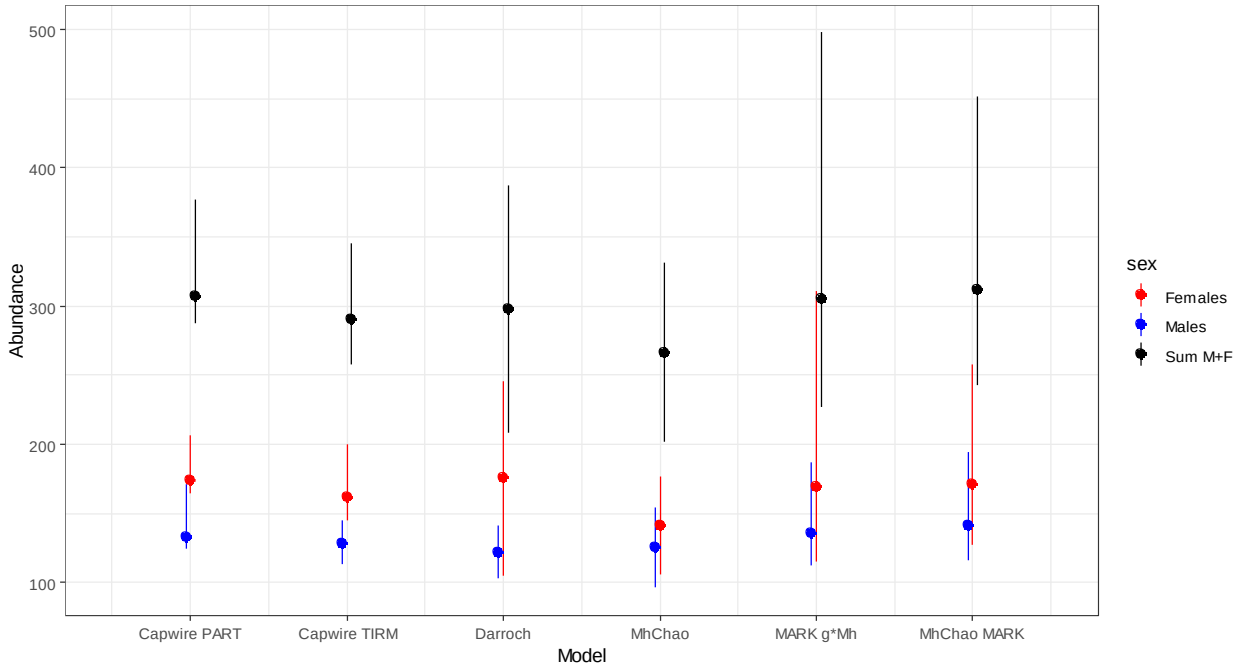

Figure 5: Mark-recapture modelling results using different modelling approaches. The "All" results mean that data for both sexes was run in a single model while "Sum M+F" means that the results for both sexes were added to provide the total estimate. The exception in this is MARK where group (sex) is explicitly included in the model. The results are for superpopulation.

We used the Capwire TIRM models fitted for each sex separately for the final estimate. Capwire performs well in small datasets (the datasets become small when we separate the data by sex) and is robust to capture heterogeneity. While the result is very close to other results (except Mh-Chao run on continuous sampling data), the confidence interval is narrower. The model is expected to be more powerful since than the other models we used since its assumptions fit better to the way the samples in this study were actually collected in the field.

3.5 WALK ANALYSIS AND EDGE EFFECT CORRECTION

Since the sampling area doesn't have natural or artificial linear barriers around it, we can expect that many of the detected bears have only a part of their homerange within. This causes the edge effect and means that we are not estimating the population within the sampling area in our mark-recapture estimates, but rather the **superpopulation** of animals from a wider area.

We can analyze the movement patterns of animals detected in the study through analysis of pairwise distances between the samples of the same animal. With this, we can estimate the contributing area (W) and calculate correction factors.

The distribution of pairwise distances by sex is presented in Figure 6. We can see that males tend to have considerably larger moves than females.

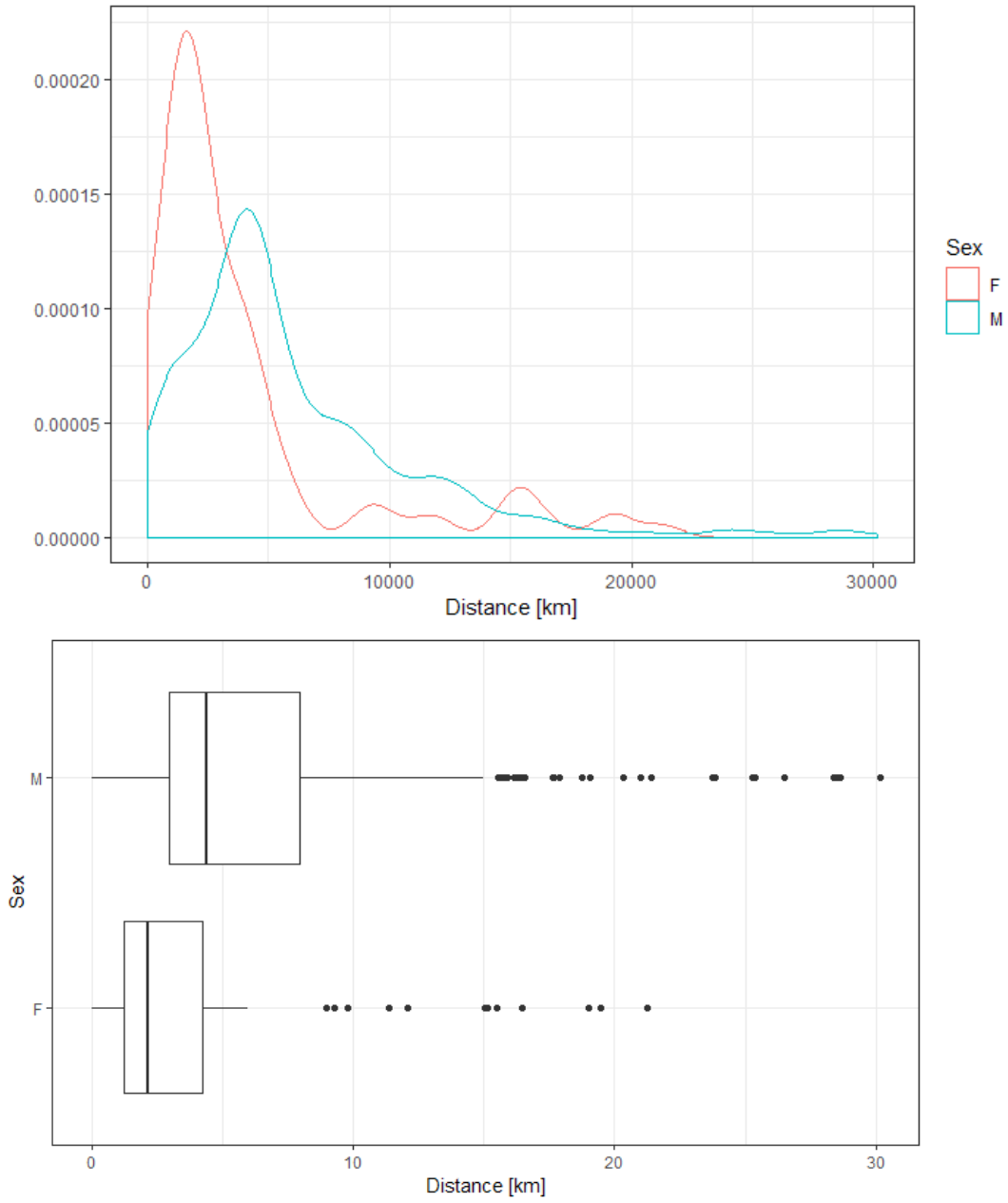

Figure 6: Distribution of pairwise distances between samples of the same animal, by sex.

However, the limited size of the sampling area means that many longer "walks" would be undetected. To correct for this, we simulated 100 000 random walks that started at the random location within the 2017 sampling area and had random length between 0 and 75 km, and checked the proportion of walks of certain length that would be detected (would end within the sampling area). This proportion was then used as a weight to calculate MMDM (see below). We removed 1% of the longest and shortest walks as outliers, and bootstrapped the entire calculation of MMDM (by randomly resampling the entire empirical walk dataset with replacement) 1000 times to obtain a more reliable mean value for MMDM and to better understand uncertainty around that mean.

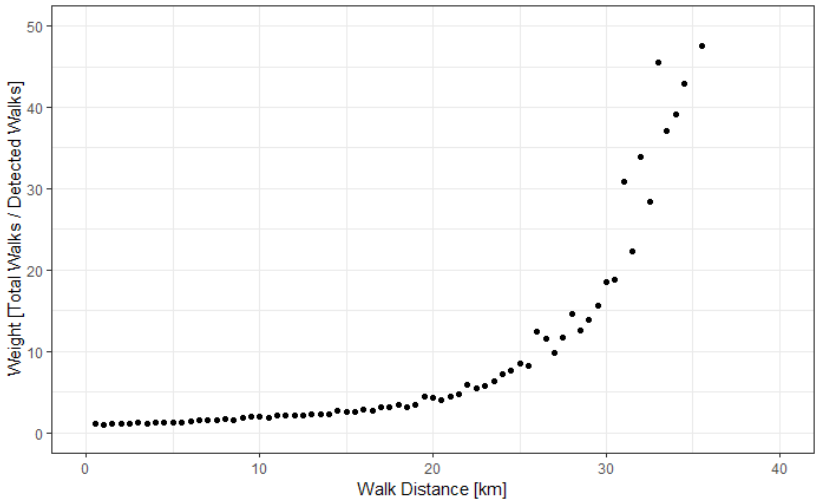

Figure 7: The walk distance weights.

The parameter used for the correction is mean maximum distance moved (MMDM), and the correction is a buffer of ½ MMDM around the sampling area. Since there are considerable differences in movement between males and females, MMDM is calculated separately for each sex (Table 5).

Table 5: Sampling areas and ½MMDM buffered contributing areas for both sexes, and edge-effect correction factors. Also included are MMDM estimates with bootstrap-determined 95 % confidence interval limits.

Name	area [km2]	min	max	MMDM [m]	min	max
Sampling Area	899	899	899			
Males Buffer	1956	1822	2093	12511	11013	14009
Females Buffer	1566	1382	1755	8059	5872	10246
CF Males	0.459	0.493	0.430			
CF Females	0.574	0.650	0.512			

3.6 LOCAL POPULATION SIZE ESTIMATES, POPULATION DENSITY ESTIMATES AND DERIVED SEX RATIO

The local population size (the number of bears expected in any given moment within the sampling area) was calculated using the correction factors for edge effect (Table 6) as the main parameter of interest in this study. We estimate that **the local population size** within the sampling area during the 2017 sampling was 152 bears (123 – 202 95% CI), 59 (49 – 72 95% CI) males and 93 (74 – 130 95% CI) females. The **population density** within the sampling area was estimated at 16.91 (8.23 – 14.46 95% CI) bears per 100 km^2.

Table 6: Mark-recapture results for superpopulation size (including bears that have parts of homerange outside of the sampling area), edge-effect corrected estimates of local population size (expected number of bears within the sampling area in any given moment), estimates of local population density and mark-recapture derived sex ratio. Confidence intervals include the uncertainty in MMDM estimation (correction of edge effect)

	N	Cid	Ciu
Superpopulation Size	290	258	345
Superpopulation Males	128	113	145
Superpopulation Females	162	145	200
Local Population Size	152	123	202
Local Population Males	59	49	72
Local Population Females	93	74	130
Population Density [bears/100 km2]			
Total Density	16.91	13.68	22.47
Density Males	6.56	5.45	8.01
Density Females	10.35	8.23	14.46
Derived Sex Ratio			
%Males	39%	40%	36%
%Females	61%	60%	64%

Using the estimated local population densities for each sex, we calculated the derived **sex ratio** as 39 % males and 61 % females.

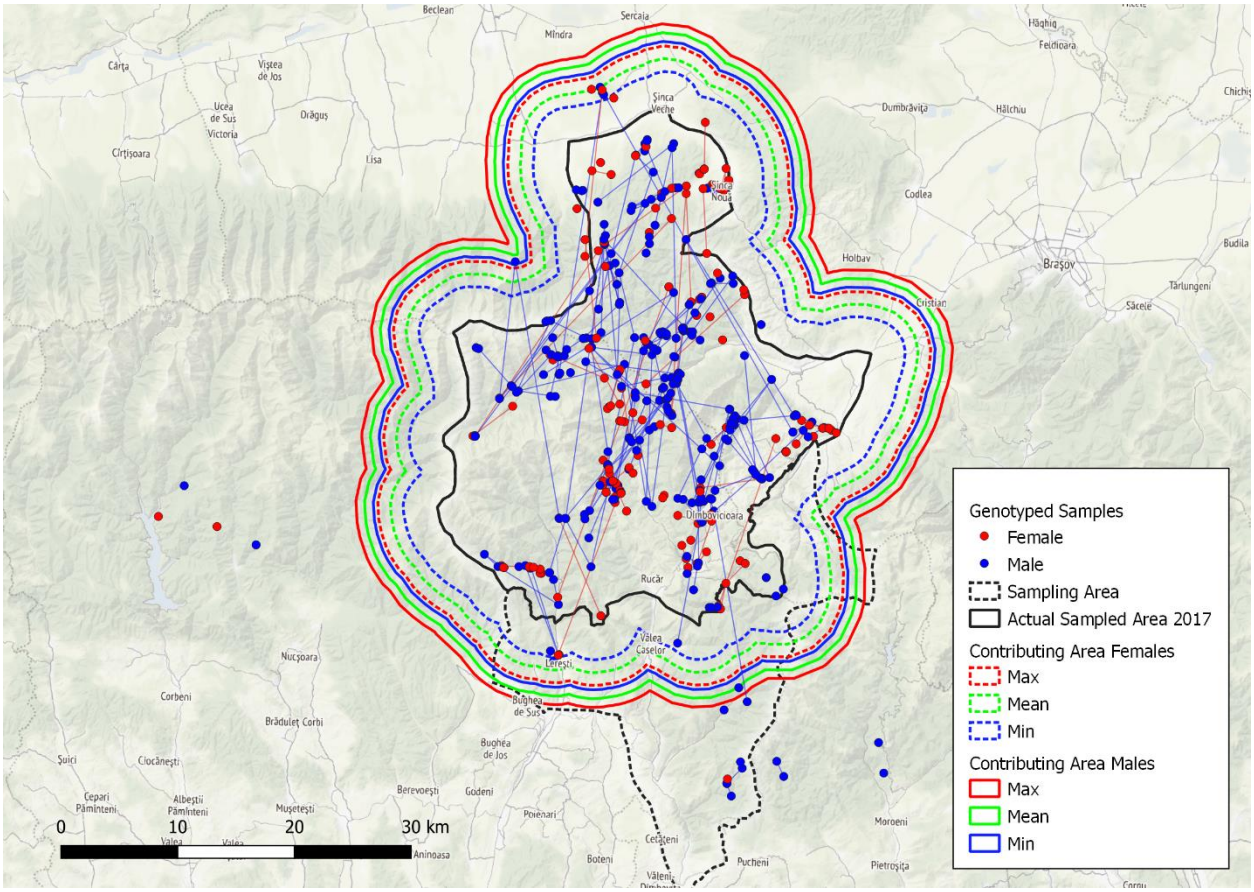

Figure 8: Genotyped noninvasive samples of brown bears, sampling area and contributing superpopulation areas for edge effect correction for each sex.

3.7 DISCUSSION

While we can certainly call the study success, it also points out some challenges that may need to be considered in future studies.

The results for population size seem robust, with several modelling approaches telling pretty much the same story. There were 173 different bears detected in the sampling within the sampling area (Table 4), which makes the final estimate of the local population size within the sampling area (the number of bears we can expect within the area in any given moment), which we estimated at 152 (123 – 202) bears, quite believable. On the other hand, looking at mark-recapture graphs (Figure 4), we can see that the graphs are not yet approaching saturation, meaning that there are still many unmarked (non-genotyped) bears in the area. This supports the high estimated number for the superpopulation (290, 258 – 345).

The population density is also high, but quite comparable to what we're observing in good bear habitat in Slovenia and Croatia (Jerina, Jonozovič, Krofel, & Skrbinšek, 2013; Tomaž Skrbinšek et al., 2017, 2019).

A particular challenge is presented by the very open sampling area, where it seems that the bear population seamlessly extends over its borders on all sides. While the test indicates that by having a short sampling season we managed to attain demographic closure of the population, the obtained

mark-recapture estimate of course applies also to the wider area around the sampling area. Because of this open edge of the sampling area, we are estimating the "superpopulation", and the problem is called "the edge effect". A new way of dealing with this problem is through use of spatially explicit capture recapture (SECR) modelling. Unfortunately, these models are designed for trap-grid type of study designs and unsuitable for the data in this study (although this will hopefully change in the future). An older approach is to use some sort of movement data (often telemetry, in our case pairwise distances between samples of the same bear) to estimate a buffer area around the sampling area from where the majority of "superpopulation" animals are coming and use this to provide a correction (Wilson & Anderson, 1985). A recent simulation study (Luštrik et al., unpublished) indicates that this correction works surprisingly well, and we used it also in this study. Since males on average have larger homeranges than females, we needed to estimate the correction for each sex separately.

An interesting effect of the edge effect and different capture probability of different sex was also observed in the sex ratio. In a (until recently) trophy-hunted bear population, one can expect the sex ratio to be skewed in favor of females. However, in this study we counter-intuitively detected more males than females. But when the actual mark-recapture estimates were done and the sex ratio derived from the calculated population densities, it was estimated to be 61 % females and 39 % males – nearly identical to what was observed in Slovenia and Croatia (Skrbinšek et al. 2018, Skrbinšek et al. 2019).

An interesting property of this study is the considerable bias in capture probability of animals of different sex. This particularly applies to hair samples, where capture probability of males is considerably larger than that of females. We can also speculate that the capture probability in hair traps is not random within males either and can as well be different for different age categories and/or social status. This indicates that hair traps, if used on their own, would provide severely biased results. This is something to be aware of in future studies and if possible direct more resources towards collecting scat samples. Although there were also more scat samples collected of males than of females, this can be possibly explained by the larger superpopulation for this sex caused by larger homeranges.

A difference between different sample types is also observed when we look at the genotyping success. Scat samples have the success rate as expected given experience from previous studies – lower than what we hoped for but expected given the somewhat higher average estimated scat age. On the other hand, the hair samples amplified very well (79%) but had a problem with mixed samples since 8.8% of collected samples were mixed genotypes of two or more individuals.

In any case, while hair traps are a useful source of samples and were of critical importance in this study, the main focus should be collection of scat samples. They require less participation of the animal, and are less biased by sex, age and behavior of different animals. This gives them much better statistical characteristics for mark-recapture analyses.

4 RESULTS AND DISCUSSION - WOLF

4.1 LABORATORY ANALYSIS AND GENOTYPING SUCCESS

We received 147 samples in the laboratory for analysis. Genotyping results are presented in Tables 7 and 8. While we managed to genotype 68.7 % of the received samples, 22 of them were either non-target species or mixed (DNA from two or more animals, in urine samples), bringing the effective success rate (proportion of useful samples) to 53.7 %

Table 7: Genotyping results, wolf samples.

Result	N	%
Genotyped	101	68.7%
- *Wolf*	*79*	*53.7%*
- *Dog*	*6*	*4.1%*
- *Fox*	*4*	*2.7%*
- *Mixed*	*12*	*8.2%*
Degraded DNA	46	31.3%
Total	147	100.0%

As expected, the proportion of mixed samples was the highest in urine samples (also in hair, but since there are only two hair samples this remains anecdotical for wolf samples). There are also mixed samples among scat samples. This is also commonly observed – we assume (and have observed in snow tracking) that another wolf or a fox would urinate on an existing wolf scat. Another possibility is that a wolf has eaten a fox, dog, or another wolf.

Table 8: Genotyping results for wolf samples by sample type. Genotyping success is proportion of samples genotyped (including mixed samples and non-target species). The effective yield is the proportion of successfully genotyped samples that had wolf DNA.

Sample Type	Dog	Fox	Genotyped	Mixed	Total Genotyped	Degraded DNA	Total	Gen. Success	Effective Yield Wolf
Blood (noninv.)			1		1	1	2	50.0%	50.0%
Hair			1	1	2	6	8	25.0%	12.5%
Scat	5	3	58	5	71	29	100	71.0%	58.0%
Tissue			1		1		1	100.0%	100.0%
Urine	1	1	18	6	26	10	36	72.2%	50.0%
Total	6	4	79	12	101	46	147	68.7%	53.7%

4.2 SPECIES IDENTIFICATION AND HYBRID DETECTION

Since wolves and dogs are closely related (sub)species, determination between them is not always straightforward. In the case of this study we could immediately determine two samples of two different animals to be dogs. The third dog (found in four samples) we included into the hybrid detection analysis. It was determined to be a dog.

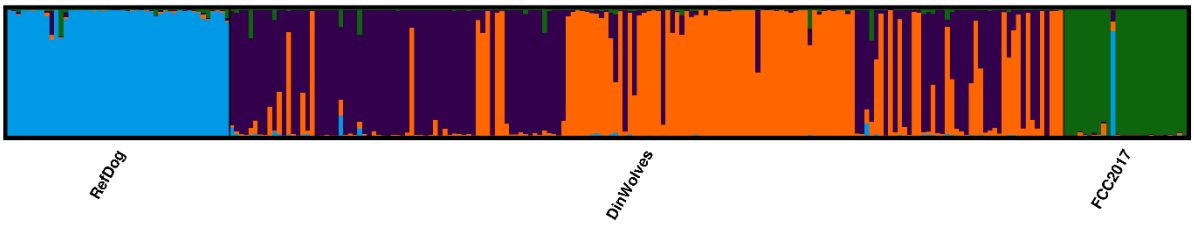

Figure 9: STRUCTURE hybrid detection analysis, K = 4. Wolf or presumed wolf samples were included in the same STRUCTURE run as reference wolf samples from the Dinaric Mountains of Slovenia and Croatia, and reference dog samples. One dog and no hybrids were detected in samples from Romania. Each vertical line is an individual, proportion of color is proportion of different cluster assignment. FCC2017 = animals sampled in this study.

The resolution of the analysis was best at K=4 hypothesis (4 clusters assumed) since there is genetic structure in the wolf reference samples from the Dinaric Mountains. Wolves from Romania are genetically clearly distinct from Dinaric wolves and form their own cluster. For future analyses it would make sense to also sample local dogs in the area (large, mixed breeds).

4.3 SPATIAL AND TEMPORAL DISTRIBUTION OF SAMPLES AND DETECTED INDIVIDUALS

Distribution of samples and genotyping results are shown in Figure 9. Most collected samples and all genotyped wolf samples were collected within the sampling area.

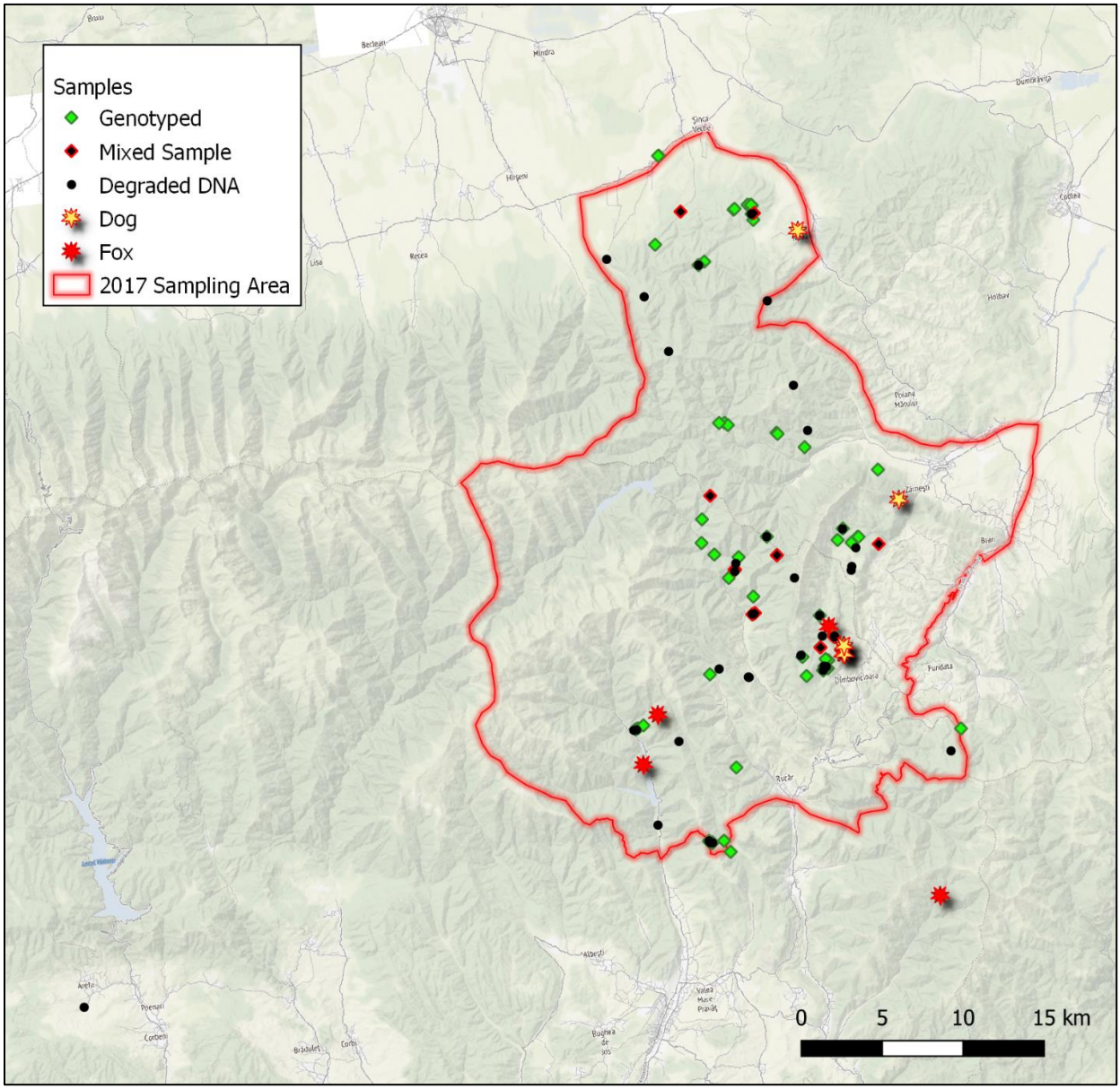

Figure 10: Distribution of samples collected in wolf sampling and genotyping results.

Figure 10 shows how samples were collected through time. All samples were collected between September 2017 and March 2018, meaning that they contain a single generation of pups which were already detectable in the beginning of sampling (in line with the critical assumption of population closure).

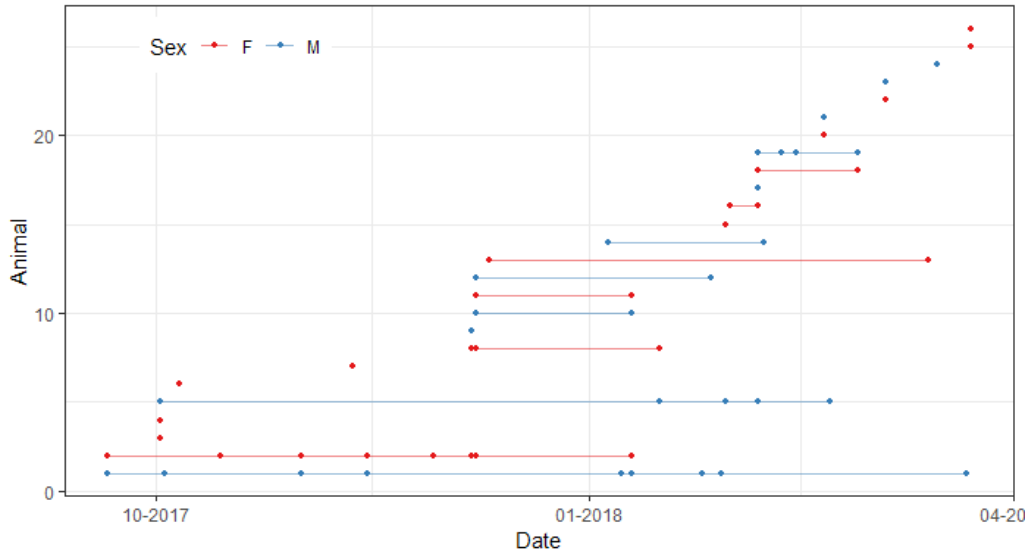

Figure 11: Temporal distribution of wolf samples. Each line is an individual wolf, each dot a sample, time goes from left to right.

Altogether, 26 different wolves were detected, 15 females and 11 males.

4.4 PARENTAGE AND PACK IDENTIFICATION

Through parentage analysis we identified four wolf packs (altogether 21 animals, 12 females and 9 males) and five animals (3 F, 2 M) for which we couldn't determine any family relations (Figures 11, 12). Apart from the single unrelated animal found in the Pack 2017_1, all unrelated animals were found at the edges of the detected packs, and many are probably members of neighboring packs that were not sampled intensively enough to allow pack identification.

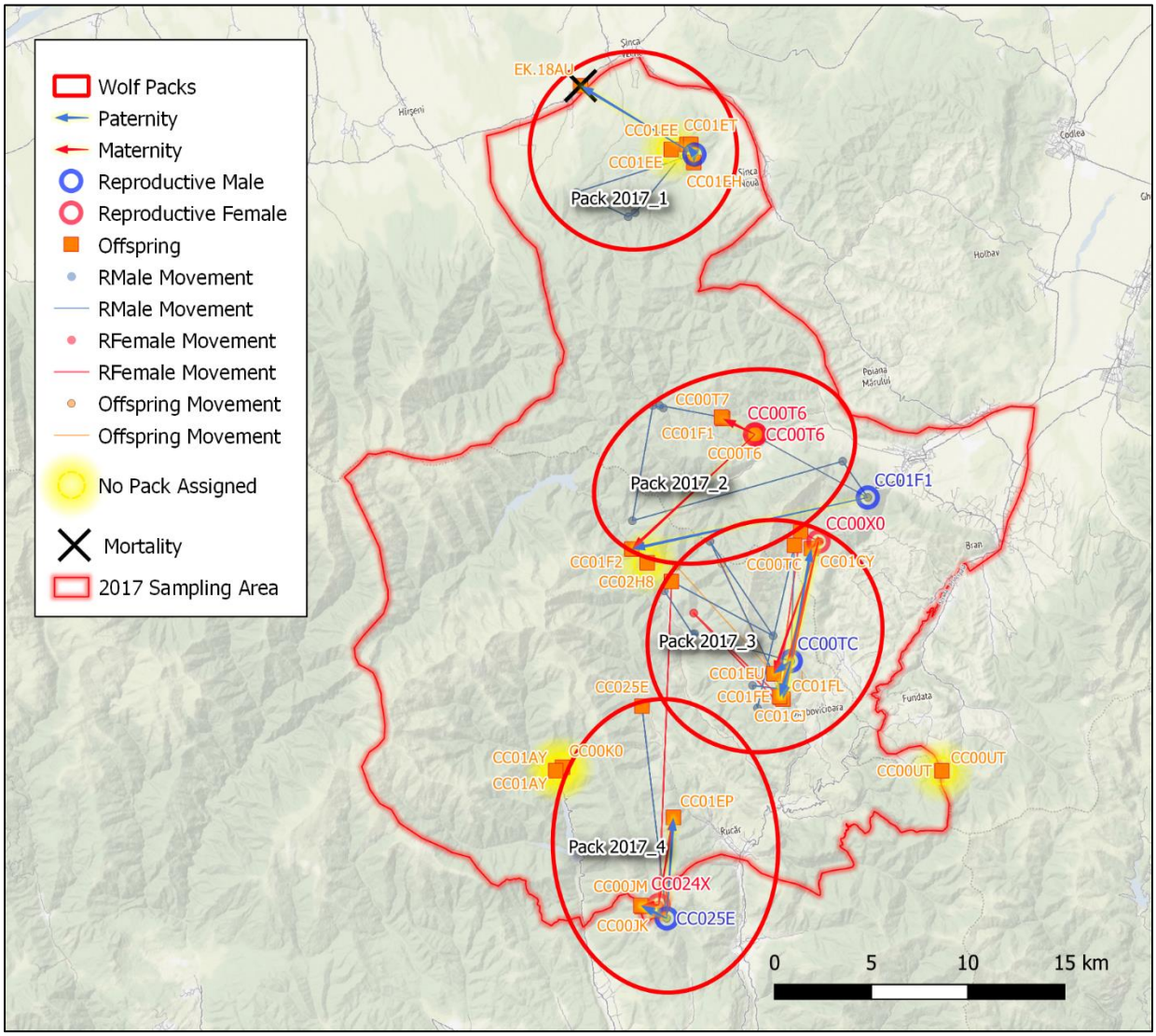

Figure 12: Distribution of wolf packs detected in 2017/2018 sampling. Apart from the animals that we could assign to packs, there are five animals for which we couldn't determine family relations.

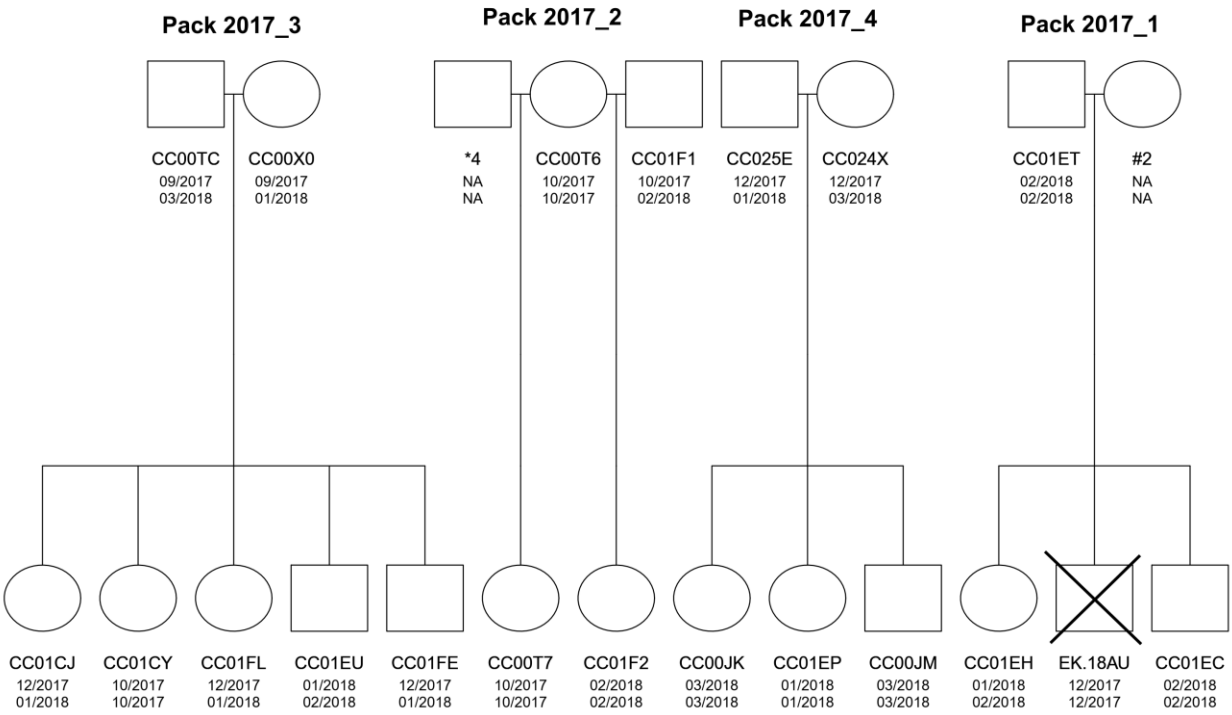

*Figure 13: Reconstructed pedigree of the detected packs. The five animals for which we couldn't determine family relations are not drawn. Square – male, circle – female. The individuals starting with # and * are unknown, the crossed-out animal is detected mortality.*

4.4.1 Pack 2017_1

This pack is in the north of the area and has suffered a detected mortality of one of the offspring during the sampling. We detected four animals, the reproductive male and three offspring (one died in a traffic mortality). We also detected one unrelated female within the pack homerange which seems to be moving with the pack (samples found at the same location on the same day as those of other pack members). Reproductive female was not detected.

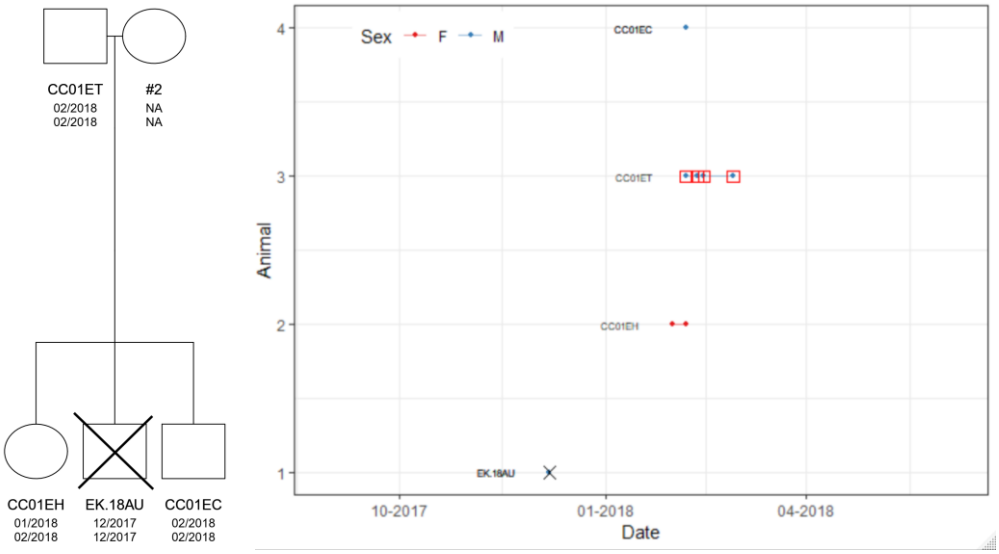

Figure 14: LEFT: Pack 2017_1 pedigree. RIGHT: Pack 2017_1, time graph. Each animal is in its own line, reproductive animals are bounded by red squares. Mortality is shown as an "X".

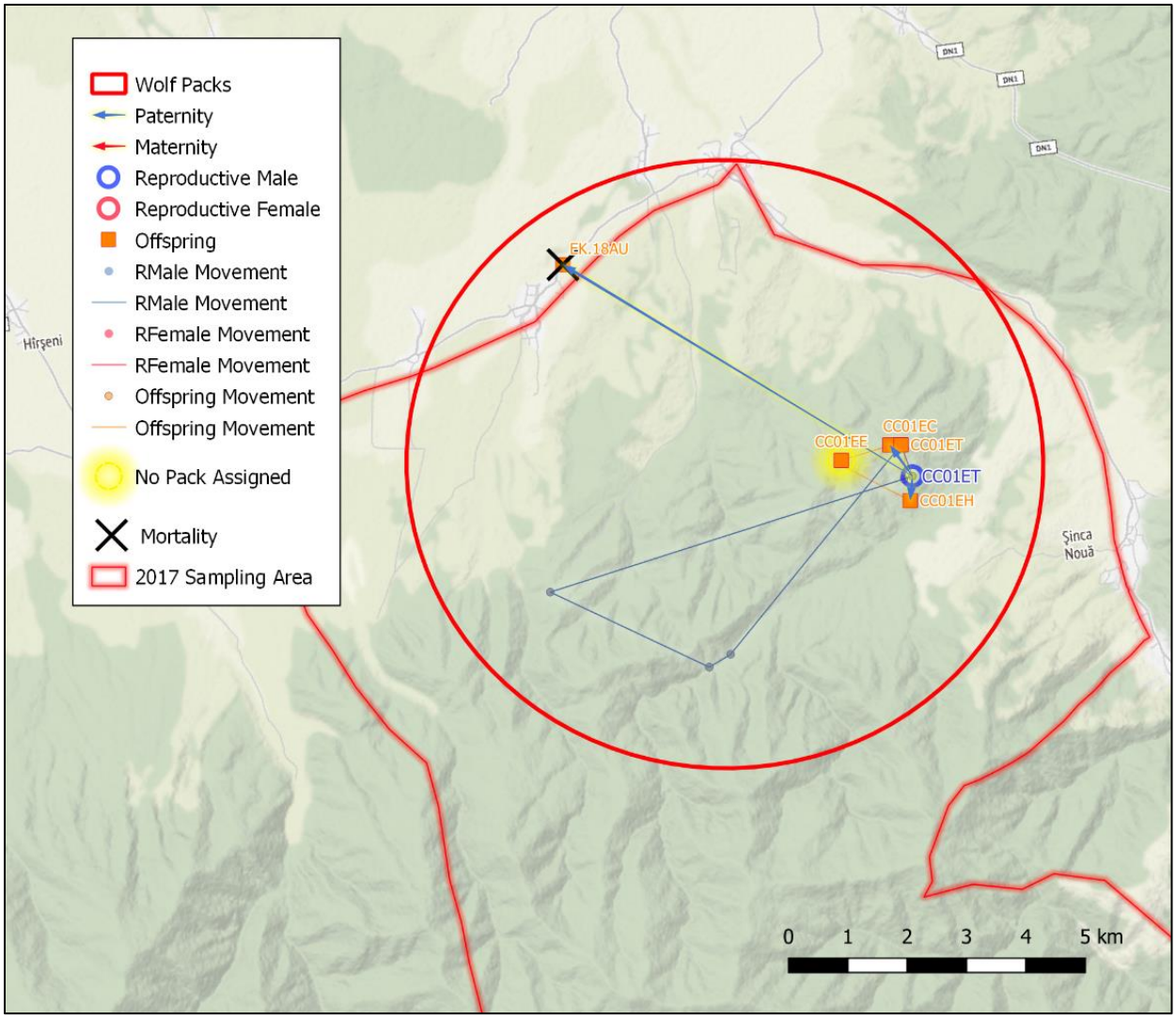

Figure 15: Pack 2017_1. The unrelated animal CC01EE (female) was detected together with the pack and seems to have joined the pack. Reproductive female was not detected in this pack.

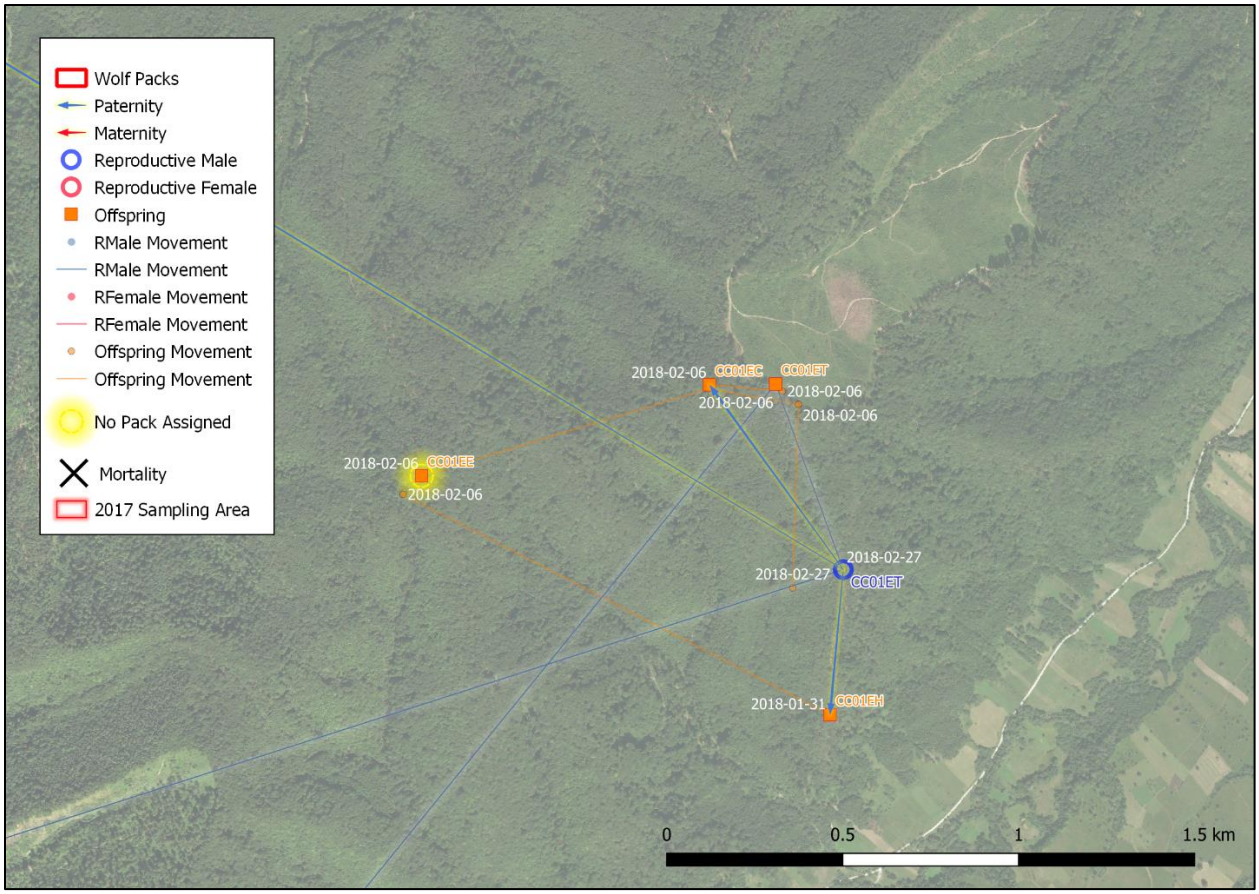

Figure 16: Pack 2017_1 close-up. Movements of the unrelated CC01EE female together with the 2017_1 pack.

4.4.2 Pack 2017_2

In this pack we detected four animals, both reproductive wolves and two offspring. A peculiarity is that one of the offspring seems to be unrelated to the reproductive male (Figure 16, Figure 17).

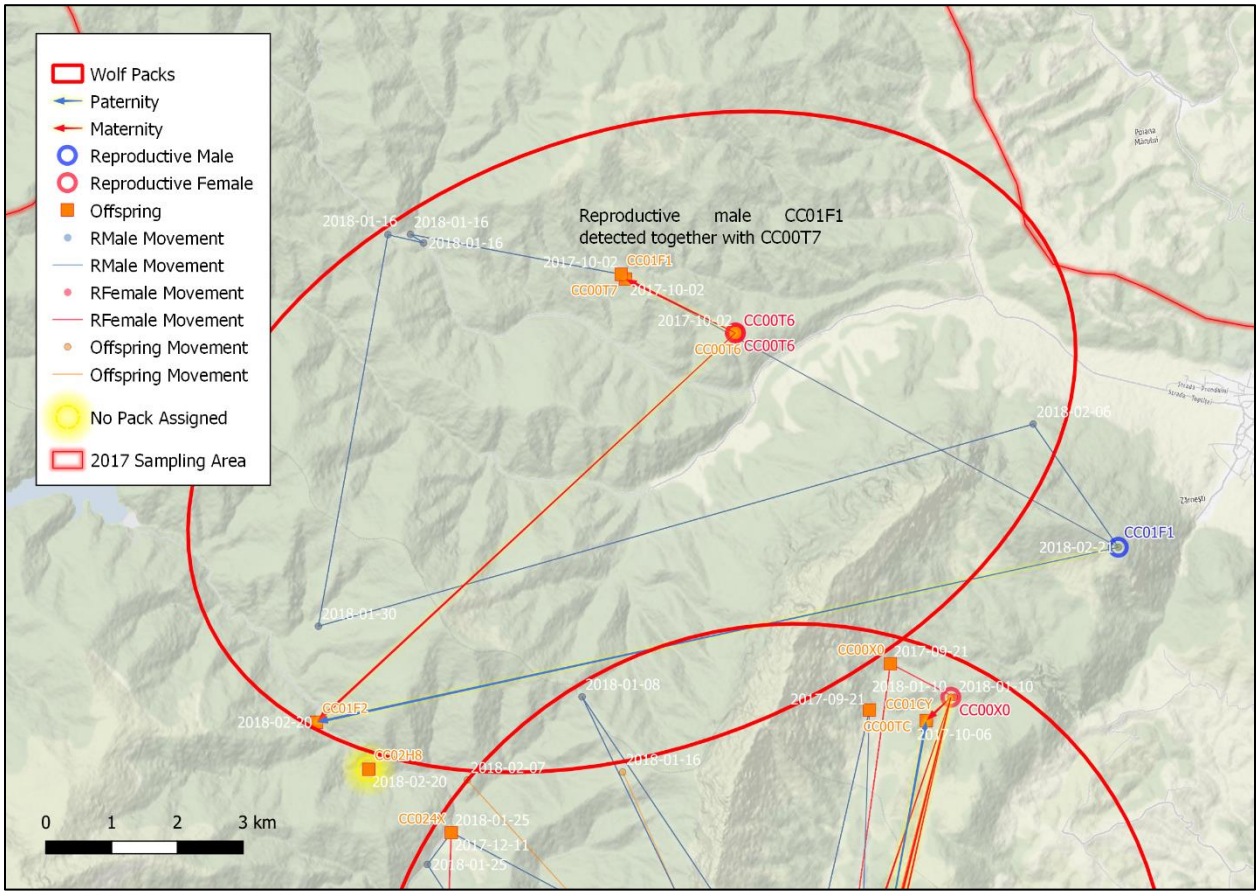

Figure 17: Pack 2017_2 map.

This could be an error in parentage assignment, but the data checks we did make this highly unlikely. We have detected a case before in Slovenia when one of the reproductive pair died and a new animal took its place with "old" pack members still remaining around if the mortality occurred when the pack had pups (in other cases a pack dissolved upon mortality of a reproductive wolf). We should be able to clarify this further if other pack members are detected in a subsequent season.

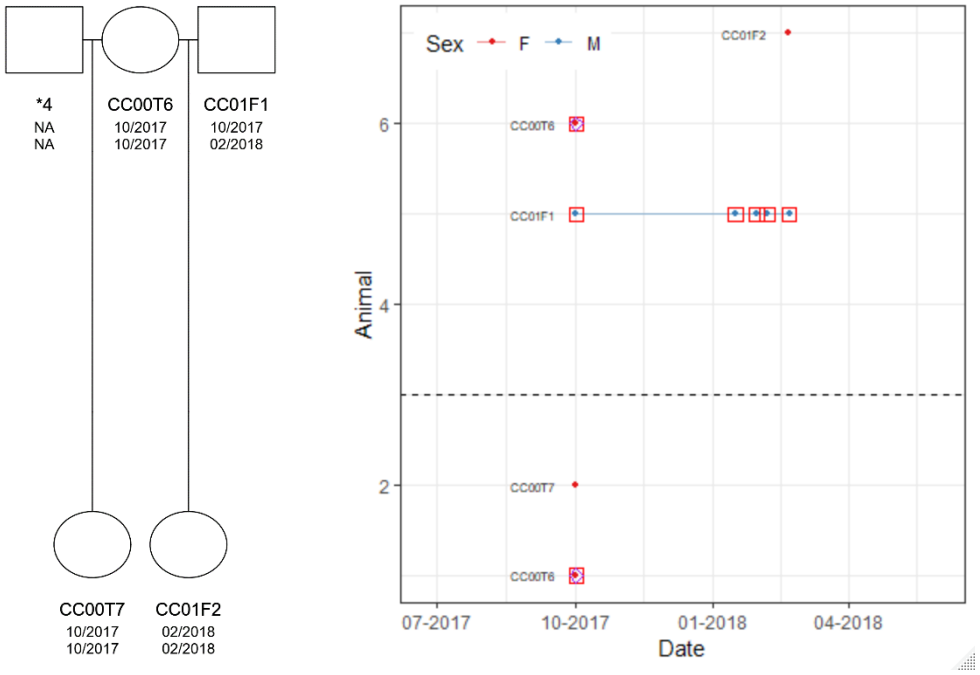

Figure 18. LEFT: Pack 2017_2 pedigree. Animal CC00T7 doesn't seem to be related to CC01F1 (reproductive male), but seems to be related to CC00T6 (reproductive female). RIGHT: Time graph. Each animal is in its own line, reproductive animals are bounded by red squares. There are two sub-families since the reproductive male is stepfather to CC00T7.

4.4.3 Pack 2017_3

In this pack we detected both reproductive wolves an five offspring, altogether seven wolves.

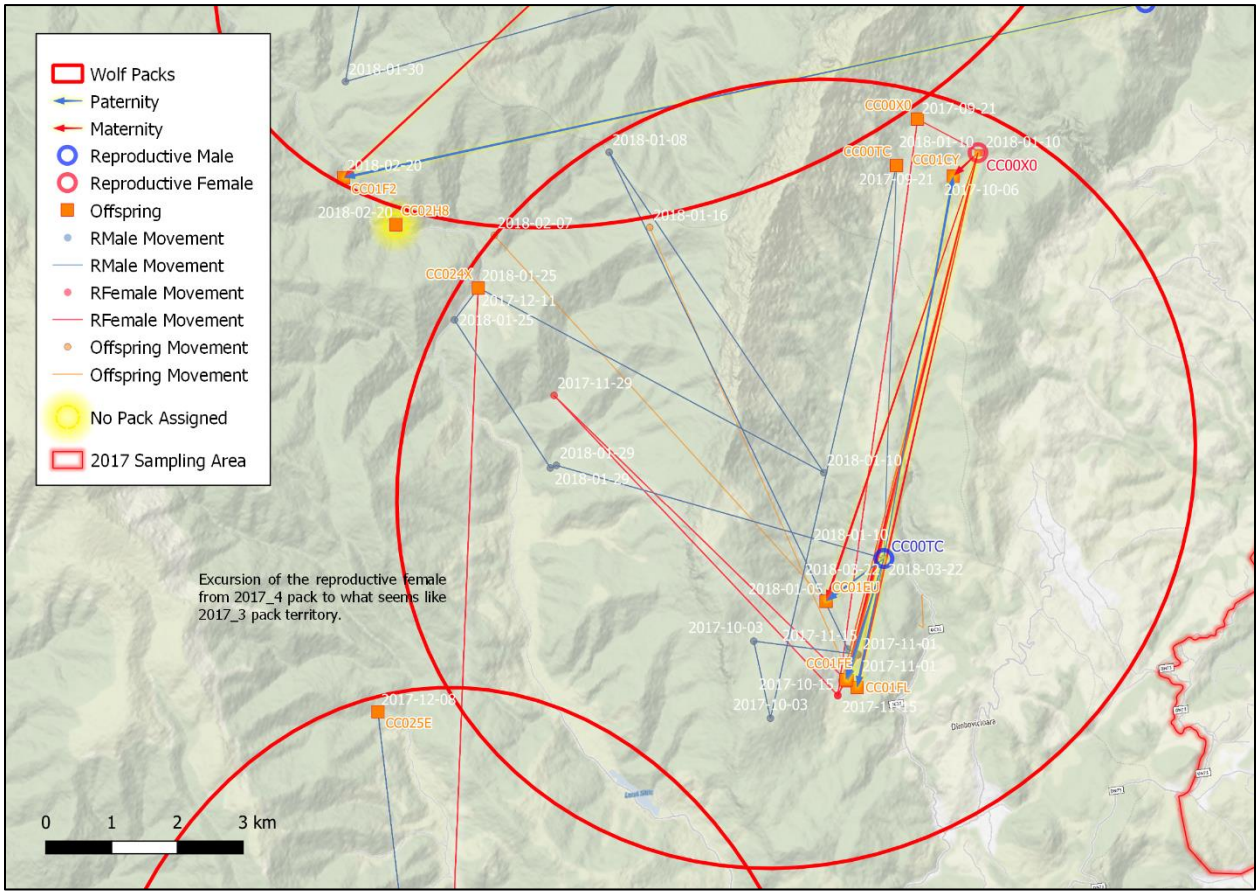

Figure 19: Pack 2017_3. This pack was very thoroughly sampled.

The pack was sampled very thoroughly – all but one animal have been recaptured, and both reproductive animals were captured multiple times.

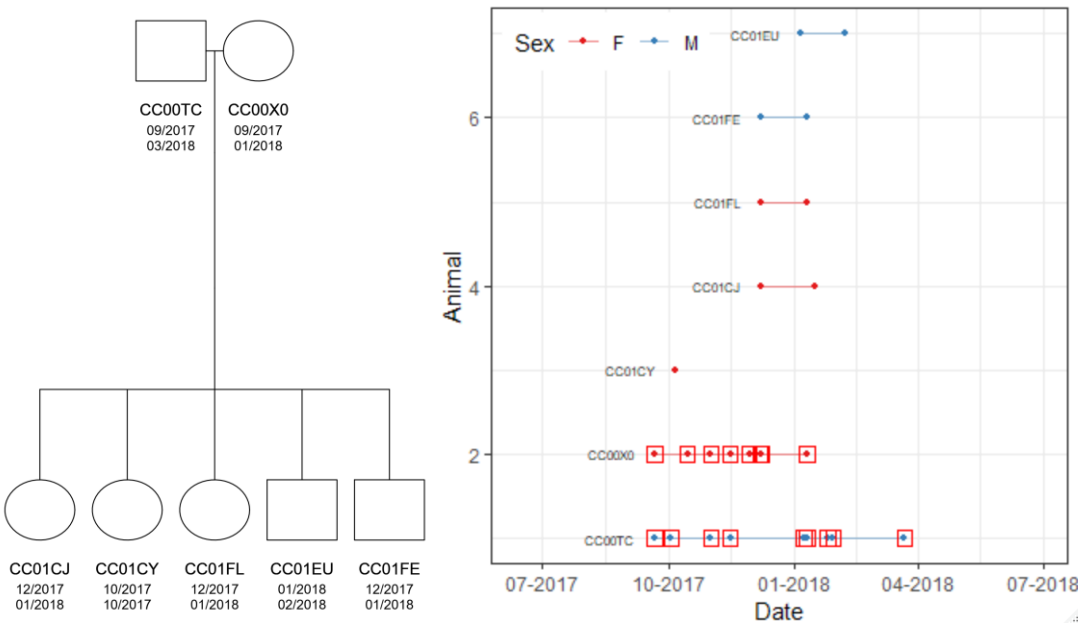

Figure 20. LEFT: Pedigree of the pack 2017_3. RIGHT: Time graph. Each animal is in its own line, reproductive animals are bounded by red squares.

4.4.4 Pack 2017_4

This pack was also well sampled. Both reproductive animals and three offspring (one male, two females) were detected.

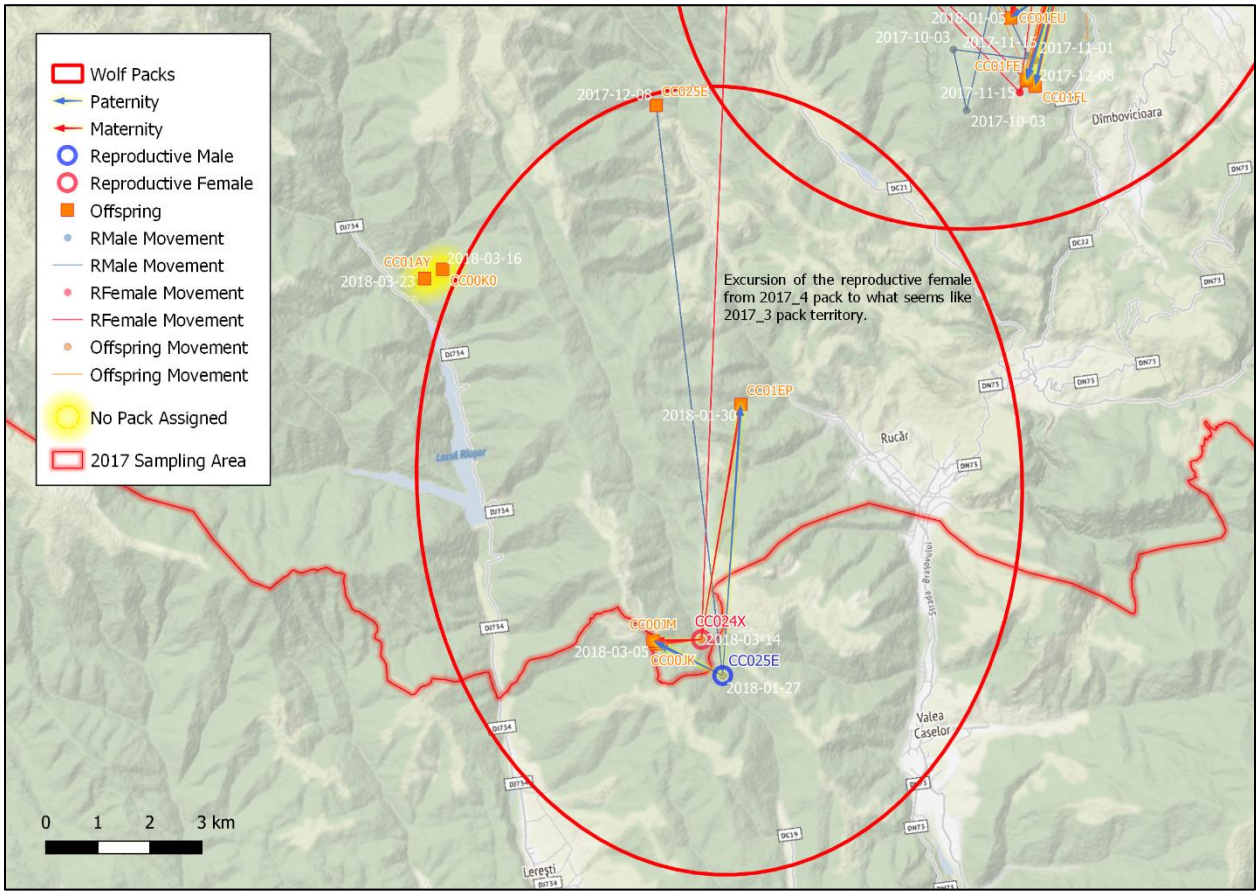

Figure 21: Pack 2017_4. Two unrelated animals to NW may be reproductive animals from the neighboring pack.

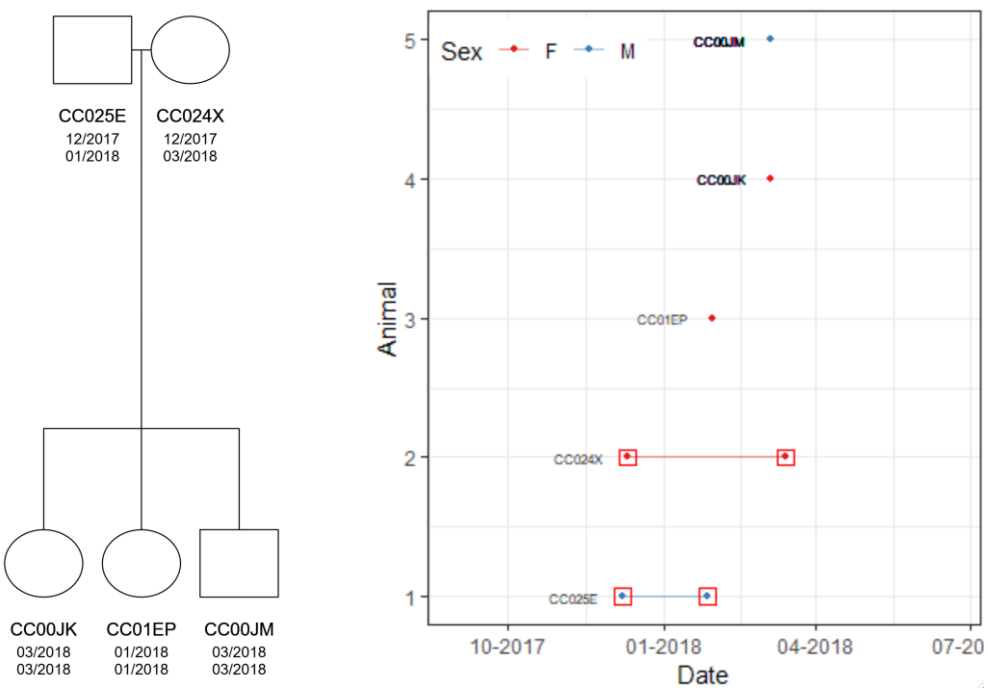

Figure 22: LEFT: Pedigree of the pack 2017_4. RIGHT: Time graph. Each animal is in its own line, reproductive animals are bounded by red squares.

4.4.5 Wolves without pack assignments

We detected five wolves (3 females, 2 males) that we couldn't assign to a pack since we detected none of their close relatives. Except for the female that seems to have been included in Pack 2017_1 in the north, the rest may be members of neighboring packs (Figure 22). For example, CC01AY and CC00K0, which were detected very close in space and time west of the presumed Pack 2017_4 territory, may be reproductive couple of the neighboring pack in that area, but we can't determine this until we've sampled their offspring.

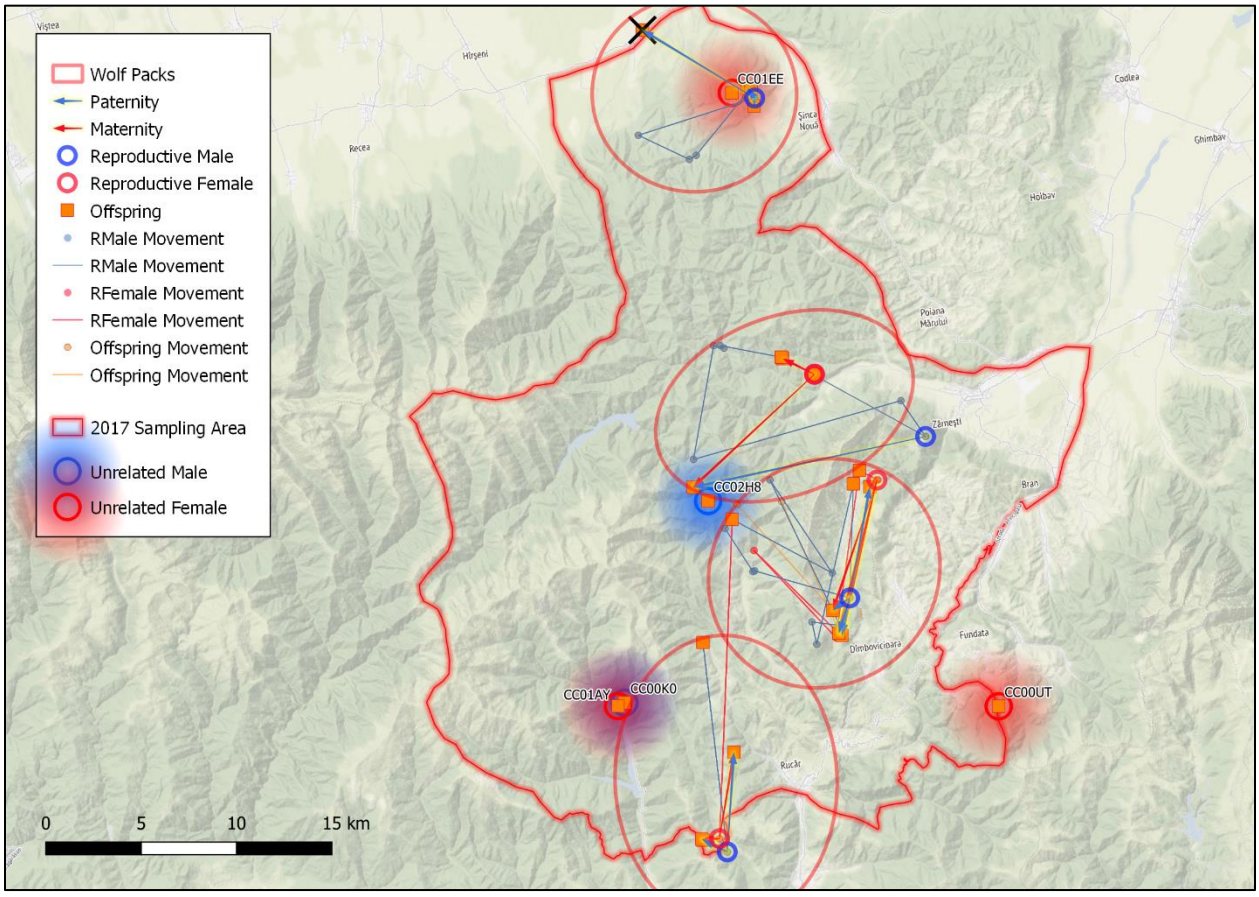

Figure 23: Animals for which we couldn't determine pack assignment.

4.5 MARK-RECAPTURE RESULTS

Although the number of samples and animals obtained in this study was low, recapture was high enough that it was possible to do a mark-recapture estimate. However, it only makes sense to include in mark-recapture the animals within the reported packs (including unrelated CC01EE which seems to be included in 2017_1 pack). The other unrelated animals were removed from the analysis. Also, the dead male in 2017_1 pack was excluded from the modelling (since it wasn't available for capture through the entire study) and later added to the estimates.

Table 9: Mark-recapture modelling results for the four detected packs of wolves (2017 - 2018)

Model	Sex	N	95% CI
Capwire TIRM	**All**	**31**	**25 - 46**
Capwire TIRM	**Males**	**10**	**9 - 12**
Capwire TIRM	**Females**	**21**	**15 - 37**
MhChao	All	32	19 - 44
MhChao	Males	11	6 - 15
MhChao	Females	22	9 - 34

We used the Capwire estimate as the final result since it should perform better in small samples than other models. We used the TIRM model for estimation – the LRT test for females was marginal ($p = 0.043$) so using the simpler ECM model was considered; however, the TIRM model was better conforming to the MhChao estimate (which provides a robust lower boundary on N) and had more conservative confidence interval.

Table 10: Actual detected numbers of animals and mark-recapture estimates.

Sex	Detected N	Estimated N	95% CI
Animals assigned to packs			
All	22*	31	25 - 46
Males	9	10	9 - 12
Females	13*	21	15 - 37
Animals not assigned to packs			
Males	2		
Females	2		

** The unrelated animal that seems to be included in 2017_1 pack was counted among the pack animals.*

As a general principle recapture rate needs to be higher in smaller studies to obtain good mark-recapture estimates, which is also why the confidence intervals of these estimates are high.

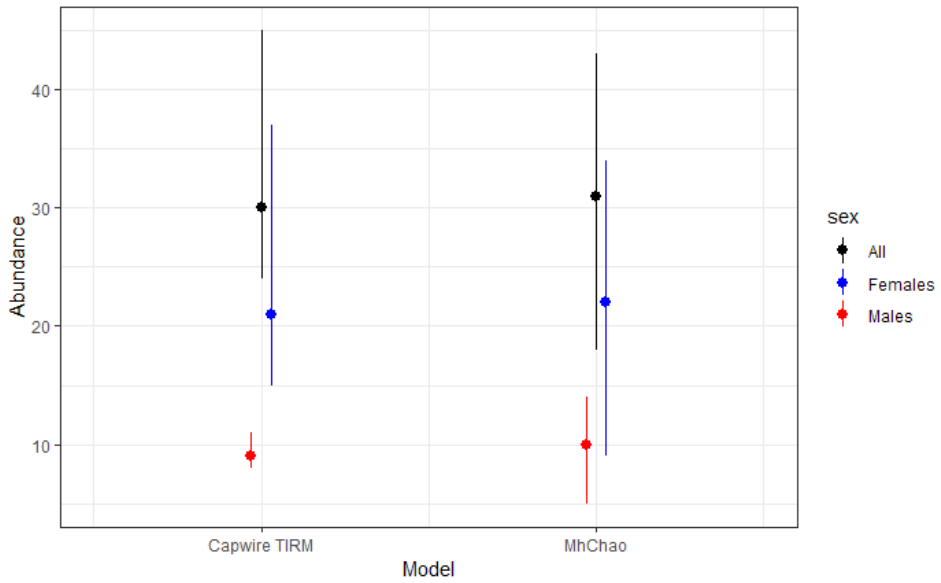

Figure 24: Graph of mark-recapture results. Confidence intervals are wide, but the results seem consistent between the considered models.

4.6 DISCUSSION

The wolf samples provided acceptable success rates. The non-target species detected are expected and difficult to avoid even with trained field personnel.

We didn't detect wolf-dog hybridization in the study area, but this is something to remain vigilant about. It would be advisable to genotype samples of local dogs as this would allow for stronger inference.

The four detected packs were reasonably well sampled, although there were no wolf samples collected in large parts of the sampling area. Considering that non-related wolves were detected, we can assume there were other packs in the area that didn't get sampled. This makes mark-recapture estimate relevant only for the area of the four detected packs. While we were able to do a mark-recapture estimate, its precision, particularly for females, is low. This is directly connected with the number of samples and total number of animals – in small studies (with small total number of animals) as was the case here there are proportionally more samples required to obtain narrow confidence intervals than in large studies with many samples.

A lot of information was obtained for the sampled packs. As the sampling will continue, this is a very good starting point for monitoring of wolf abundance, population dynamics and behavior in the area.

5 LYNX – RESULTS AND DISCUSSION

5.1 SAMPLES AND GENOTYPING SUCCESS

Altogether we received 24 samples of lynx, 3 scat samples, 17 hair samples and 4 urine samples.

Table 11: Results of genotyping of putative lynx samples.

Sample Type	Wolf	Wildcat	Fox	Lynx	Total Genotyped	Degraded DNA	Total	Genotyping Success	Effective Yield Lynx
Hair				3	3	14	17	17.6%	17.6%
Scat		3			3		3	100.0%	0.0%
Urine	1		1	1	3	1	4	75.0%	25.0%
Total	1	3	1	4	9	15	24	37.5%	16.7%

From only 3 samples we were able to obtain a reliable lynx genotype, and another sample was amplifying but the DNA was of too low quality for reliable individual ID. All three reliable samples were from different lynx.

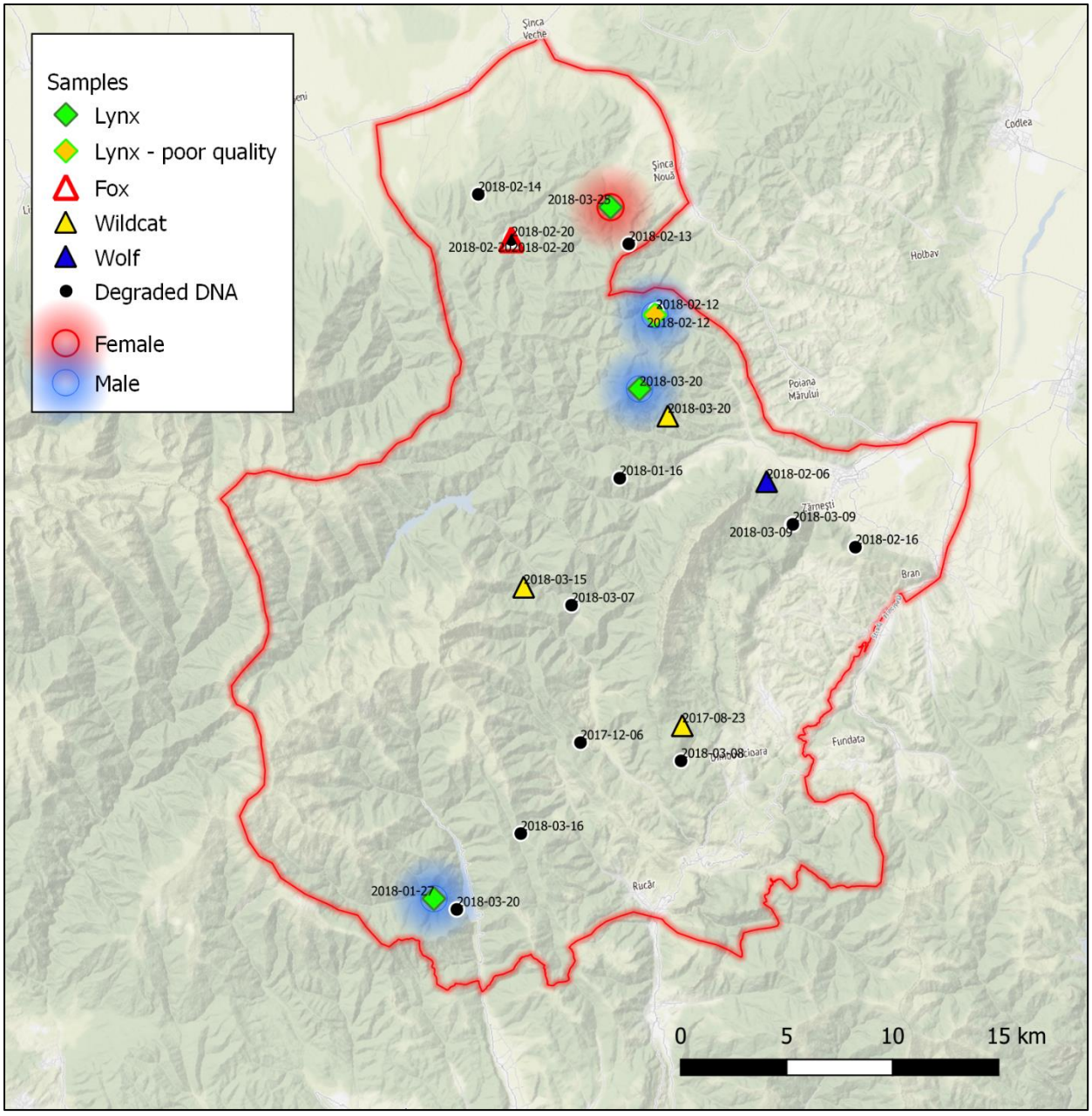

Figure 25: Results of genotyping of lynx samples.

5.2 Discussion

Genotyping success rates for lynx hair samples was extremely low. Since these samples were in majority, the overall genotyping success rate is low. For the other sample types and samples that did work, there were multiple non-target species captured – wildcat, fox and wolf (wolf sample was used downstream in wolf analysis).

Based on these data we can only say that there are at least three lynx in the area, one female and two males. The poor sample was also male, but it's impossible to determine if it's the same animal as one of the other males.

In general, of the species studied in this study, lynx is the most challenging for genetic monitoring as viable samples are difficult to obtain. There are a couple of suggestions we can make regarding the field collection protocols that may increase the success rates and the number of collected samples:

- Improve the design of hair trap. Most samples had a very small number of hair roots, which contain genetic material (median = 3 hair roots per sample, mean = 3.4). This may be the most critical issue since the higher number of hair roots in a sample typically considerably increases the genotyping success. A simple and effective hair trap has been suggested (McDaniel, McKelvey, Squires, & Ruggiero, 2000) and slightly modified in another study (Schmidt & Kowalczyk, 2006).

- Intensive snow tracking, especially during the mating season. During the mating season territorial lynx mark their territory intensively with urine, and often several urine samples can be obtained when following a single track for some distance. If this approach is taken, the samples collected while snow tracking a single track (known to belong to the same animal) should be clearly labelled as such so that the samples are not considered to be independent. An additional benefit of intensive snow tracking is that the family groups (females with cubs) can be surveyed (although during the mating season the family groups often start dissolving) and marking spots for lynx for setting hair traps and photo traps can be located.

- Setting of hair traps on places known to be used for marking by lynx (Schmidt & Kowalczyk, 2006). Lynx tend to mark the same objects (even different lynx in different years), possibly because they find them "interesting" and since by marking such objects they can more efficiently transmit their chemical message to other lynx that will also get visually attracted to the same features. It also makes sense to put camera traps on such places to possibly connect the image (coat pattern recognition) data with genetic identification and use both data types synergistically.

- Increase frequency of hair traps visits. DNA degrades fast in the environment, so the samples should be collected as soon as possible. Most studies found that 10-14 day revisits are sufficient (and at this time the hair traps can also be re-baited with the attractant).

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