Report on monitoring wolf using non-invasive DNA sampling in the Romanian Carpathians

18.07.2023

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Chapter 1
Introduction

📷 *Daniel Bîrloiu*

Monitoring large carnivore population parameters and trends is fundamental for responsible management strategies (Takinami *et al.*, 2021), particularly for shifting the management paradigm from one of controlling populations to that of human-large carnivore coexistence (Bergstrom, 2017). Population estimates guide scientists and practitioners to build coexistence actions, establish interventions and review their efficiency in practice (Lorand *et al.*, 2022), calibrate lethal management approaches when they are jeopardizing coexistence goals (Fernández-Gil *et al.*, 2016), and tempers the blame put on large carnivores in public and political discourse (Neagu, Manolache & Rozylowicz, 2022). Coexistence policies vary across the range of apex predators, from well-informed decision-making to reactive, emergency management. The informed management approach is supported by science-based population estimates (Can *et al.*, 2014), population data being transparently used to engage with relevant stakeholders (Redpath *et al.*, 2017). Metrics such as population size and density are obtained through statistical modelling from systematically collected field data,

long term data allowing to assess population dynamics especially at time of major management shifts (Creel & Rotella, 2010; Schmidt *et al.*, 2021; Takinami *et al.*, 2021). Such data is used not only to reduce present conflicts but also to address the root cause of the problem and to strengthen future coexistence opportunities (IUCN, 2019). Absence of objective population data can easily translate into poor decision-making, especially with administrations not accustomed to integrating scientific evidence when responding to socio-environmental challenges (Artelle *et al.*, 2014). The reactive management approach relies almost exclusively on urgent responses to compensate for damage produced by large carnivores and to stabilize public perception. It usually has poor data available at hand (Popescu *et al.*, 2016), and in such systems, the expertise of different stakeholders involved in large carnivore conservation is not advantageously used via institutional collaboration (Hartel *et al.*, 2019).

The wolf (*Canis lupus*) is an apex predator with a strong importance in the top-down cascade effect of the trophic levels (Vucetich, Peterson & Schaefer, 2002; Hoeks *et al.*, 2020). Usually, wolves live in packs that cover well-defined territories (Mech & Boitani, 2003). The genetic structure of the wolf population is diversified through the dispersal of individuals between connected packs (Mech & Boitani, 2003). Habitat loss, however, determined spatial variation in the genetic diversity of wolves. Across Europe, for example, heterozygosity decreased when comparing populations inhabiting compact forests of north-eastern Europe to those living in the highly fragmented Iberian Peninsula (Hindrikson *et al.*, 2017). Here, wolf-dog hybridization became a major threat to wolf conservation and although the problem is recognized as being widespread across many areas, it is alarmingly unaddressed in practice (Salvatori *et al.*, 2020). Most European countries, especially in the eastern part, lack hybridization monitoring programs to base their conservation policies on (Stronen *et al.*, 2022). Whilst wolves cause damage to livestock in the human-dominated landscape, farmers too can exert pressure through the illegal killing of wolves (Chapron *et al.*, 2014). In the wake of socio-economic changes, coexistence between farmers and wolves degrades even in traditional agricultural landscapes, mainly due to loss of traditional knowledge (e.g., not using appropriate shepherd dogs; Kikvidze & Tevzadze, 2015). Also local hunters and game managers can compete with wolves for ungulate species (Skogen, 2022), and even protected areas administrators oppose the recent range expansion of wolves as impact on charismatic species is expected (https://www.hogeveluwe.nl/en/discover-the-park/nature-and-landscape/wolf). These complex interactions have political echoes as well. In Germany, negative perception of wolves is correlated to extremist political views (Von Hohenberg & Hager, 2022). With such a complex animal, understanding pack structure and dynamics has strong ecological and management relevance. Pack structure dynamics can be linked with natural dispersal in a connected landscape, but also with human-caused mortality outside of protected zones (Rutledge *et al.*, 2010). Established packs, for example, are known to cause less damage on livestock compared to packs that are at risk of frequent member losses (Wielgus & Peebles, 2014). Wolf dispersers too were documented to predate more on livestock across agricultural areas compared to resident wolves established in areas with natural vegetation and strong ungulate populations (Mayer *et al.*, 2022). Moreover, killing wolves at a predated ranch in the Upper Peninsula of Michigan, USA, not only failed to resolve the issue, but exacerbated it by causing the packs to destabilize and expand into neighboring ranches in search of easier prey elsewhere (Santiago-Avila, Cornman & Treves, 2018).

In Europe, the Romanian Carpathians represent a stronghold for apex predator conservation and coexistence thanks to the large carnivores' community being intact and historically coexisting with humans in the traditional agricultural landscape around the mountains (Iosif *et al.*, 2022). Romanian Carpathians are expected to sustain a large number of wolves (up to 3000; Convention on the Conservation of European Wildlife and Natural Habitats, 2022) with high genetic variability being documented, suggesting good connectivity among subpopulations (Ericson *et al.*, 2020; Jan *et al.*, in submission). Climate change, habitat loss, and shifts in the wildlife management approach bring new and more complex coexistence challenges (König *et al.*, 2020).

Changes in the traditional agricultural land use have potential to bring humans and wolves closer, with land abandonment increasing in less attractive areas and rapid human expansion into forest habitats at touristic hotspots (Mustățea & Pătru-Stupariu, 2021). Development of high traffic roads forecasted for the next decades reiterate the importance of understanding population trends and distribution in order to ensure long term connectivity goals (see Fedorca *et al.*, 2019 for an example on Carpathian brown bears *Ursus arctos*). In 2016, the Romanian government banned the trophy hunting of wolves, which was historically managed by hunters and game wardens through hunting quotas and supplementary feeding. Supplementary feeding was usually targeted to other species but carrion feeding attracted wolves as well. But this decision was followed with minimal effort to involve these stakeholders in the species conservation and sustain their interest in protecting and monitoring wolves (Riener, 2019). In this dynamic context, we lack science-based monitoring initiatives to provide estimates on wolf population and data on pack structure. Across the Romanian Carpathians, approximately 67,000 km^2, a single pilot monitoring study exists, covering 4000 km^2 in the Eastern Carpathians. The results revealed detailed data on packs and pairs distribution through systematic non-invasive DNA sampling (Gazzola, Sin & Corradini, 2017).

Non-invasive DNA sampling, with DNA extracted from hair, scat or urine deposited by animals in their habitats has emerged as an effective tool in conservation and coexistence science (Schwartz, Luikart & Waples, 2007). This approach makes the sampling of animal populations, especially elusive large carnivores, significantly faster and more cost-effective and can be better integrated into monitoring programs generating long-term data (Kelly *et al.*, 2012). Non-invasive DNA sampling is currently used for detailed inferences on sex ratio, abundance and density in many large carnivores, including the Amur leopard *Panthera pardus orientalis* (Cho *et al.*, 2022) and brown bear (De Barba *et al.*, 2017). In the case of a social animal such as the wolf, the genomic approaches provide even more detailed inferences on family relations and hybridization with domestic dogs (Stronen *et al.*, 2022). Caniglia *et al.*, (2014) for example, collected non-invasive DNA samples for nine years to determine genetic variability and integrity of the wolf population, pack structures and wolf-dog hybridization in the Apennines in Italy. Besides genetic variability and pack dynamic, genetic detections of wolves can be modelled through capture recapture models to estimate population size parameters, with scientists initially using non-spatial models for this purpose (Pollock, 2000; Williams, Nichols & Conroy, 2002). However, for non-spatial models, uncertainties around estimating the effective sampling area remain a concern when studying a species with high movement potential in compact or well-connected ecosystems (Hupman *et al.*, 2018). A new approach for population estimates has emerged in the family of spatially explicit capture-recapture models (hereafter SECR; Royle *et al.*, 2014). SECR models address the bias induced by the edge effect (i.e., quantified as the exchange of individuals with the highly suitable habitat from outside of the established sampling area, individuals that were sampled only occasionally as they entered the sampling area). López-Bao *et al.*, (2018) used SECR modelling to estimate wolf population size and to assess spatial patterns of wolf density.

In this study, we focused on estimating demographic parameters such as abundance and density, as well as assessing packs composition and dynamics in a wolf population from Southern Carpathians, Romania. We collected non-invasive DNA samples of scat, urine on snow, and hair to trace individual wolves and families across three consecutive years. We reconstructed packs by combining parentage analysis using genetic data with field observations. We estimated abundance using non-spatial capture recapture modelling (CMR) and estimated population density by acknowledging the edge effect uncertainty in a SECR modeling. We assessed spatial patterns of wolf population density by integrating sampling effort in the SECR model. Considering the scarcity of such monitoring in Romanian Carpathians, we also provide recommendations for an efficient monitoring scheme of wolf population with specific protocols for the Romanian Carpathians such as sampling size, tracking sampling effort, genotyping success, sampling area and modeling parameters.

Chapter 2
Sampling area

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The study area is located in Southern Romanian Carpathians, including parts of Făgăraș Mountains, Iezer-Păpușa, Piatra Craiului and Leaota Mountains, ranging in altitude between 600 and 2400 m (Figure 1). It includes a national park and overlaps with four Sites of Community Importance managed under the Europe's network of protected areas, the Natura 2000 network. Deciduous, coniferous, and mixed forests in equal proportions cover 62% of the area. Mixed forests are located at mid-altitudes (around 1400 – 1500m a.s.l.) with species composition dominated by beech-fir or beech-fir-spruce *Fagus sylvatica*, *Abies alba* and *Picea abies*. Beech dominates at lower elevations (900 – 1300 m), and conifers at higher elevations (1500 – 1800 m). Above 1300 m, the ecosystem mixes transitional woods with *Pinus mugo*, *Vaccinium subsp.* shrubs and alpine grasslands. Lowlands are characterized by small-scale farming and traditionally maintained mosaic-like landscapes (pastures, hayfields, and forests). Tourism is growing in the accessible areas, while livestock grazing historically shaped the alpine ecosystems and is still common nowadays. In the last three decades, the area was affected by forest clear-cut operations (Kuemmerle *et al.*, 2009), with logging still being an important economic activity. The study area harbors an intact mammal assemblage, including large and meso-

carnivores, such as brown bear (*Ursus arctos*), wolf (*Canis lupus*), Eurasian lynx (*Lynx lynx*), wildcat *(Felis silvestris);* fox (*Vulpes vulpes)* and badger (*Meles meles*) and their prey: roe deer *(Capreolus capreolus),* red deer (*Cervus elaphus*), chamois (*Rupicapra rupicapra*), wild boar (*Sus scrofa*) and leporids (e.g., hare, *Lepus europaeus*). European bison (*Bison bonasus*) and Eurasian beaver (*Castor fiber*) have also been recently reintroduced to the study area (https://www.carpathia.org/wp-content/uploads/2022/11/FCC_Raport-anual_EN_2021_FINAL-compressed.pdf).
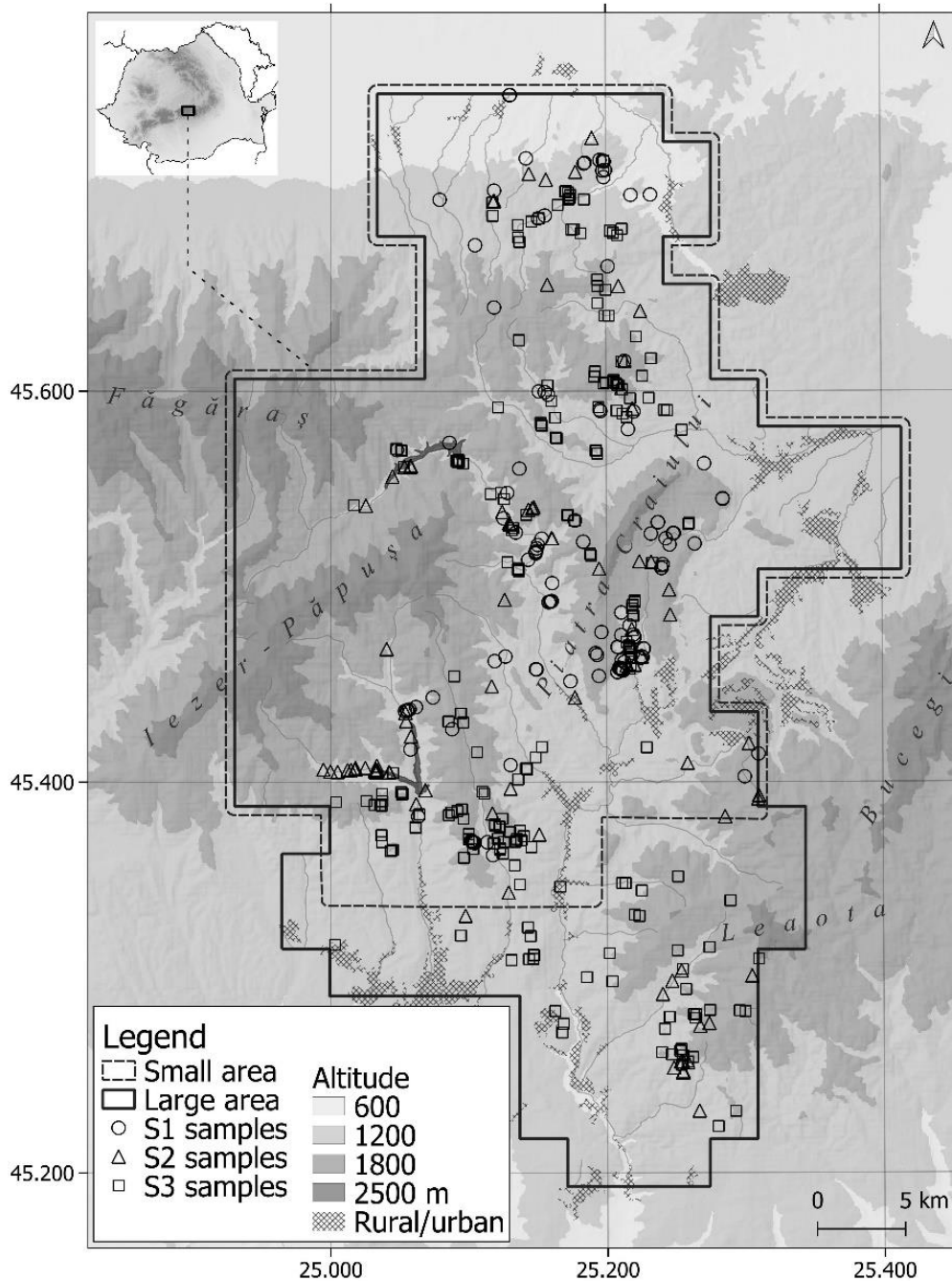

Figure 1. Sampling area for non-invasive DNA monitoring of wolf (Canis lupus) in Southern Carpathians, Romania. Map shows different symbols for the small and large sampling areas covered in different sampling sessions. In session 1 we sampled an area of 1100 km² (S1sa) and the resulting data was used for reconstructing the wolf packs and for estimating demographic parameters. In session 2 the same area was sampled, but we used the data only to reconstruct wolf packs (S2sa). In session 3 the sampling area was expanded to 1400 km² (S3la) and we used the data for reconstructing the wolf packs and for estimating demographic parameters.

Chapter 3
Methods

📷 *Ruben Iosif*

3.1. Sampling design

The sampling was conducted over three sessions: 1 July 2017 – 31 June 2018 (Session 1), 1 July 2018 – 31 June 2019 (Session 2) and 1 July 2019 – 31 June 2020 (Session 3). Although the sampling was open continuously from July to June in each session, from November to May samples were collected systematically using snow tracks to increase sampling success, with only opportunistic sampling from May to November. All samples were genotyped and used to identify individuals and reconstruct wolf families, while only systematically collected samples were used for estimating demographic parameters. In Session 1 and Session 2 an area of 1100 km^2 was sampled, an area smaller than in Session 3, that hereafter will be referred to with a *sa* subscript, i.e., S1$_{sa}$. In Session 3 the sampling area was expanded at the southern tip resulting in a larger area of 1400 km^2, hereafter referred to with a *la* subscript, i.e., S3$_{la}$) (Figure 1).

Prominent ridges, rivers (and adjacent logging roads), tourist routes, and visible animal paths were the primary target areas when searching for samples. If fresh tracks were observed in the snow, the trail was backtracked

until samples were found. The genetic material from non-invasive DNA samples was usually collected from scat, urine and hair. More rarely, it was collected from blood on snow, saliva on carcasses of predated ungulates, and tissue samples from dead wolves. Sample age cutoff was a maximum of 5 days; any samples estimated to be older were not collected as the expected genotyping success for older samples is low. While sample age estimates are subjective, they have shown to be an excellent predictor for the expected genotyping success (Skrbinšek, 2020).

The hair sampling kits consisted of a paper envelope with 10 g of Silica gel. The scat sampling kits consisted of 8ml tubes, and the urine kit consisted of 50 ml tubes. Both tubes were filled with 96% ethanol to preserve the sample. The scat and urine kits also contained two wooden sticks that were used to collect the samples from the surface of the scat or from the snow. A secondary function of the sticks was to mark that the sample had been collected to avoid accidental re-sampling in the field. Sampling kits were packed in resealable bags fitted with labels to record the collector's name, date, GPS coordinates and the field-estimated age of the scat. They had stickers with unique ID codes, which identified individual samples and connected the laboratory results with the field data. In a dedicated mobile app, we collected data on GPS location, habitat description, scat content, origin of a collected urine or hair (i.e., from the side of the road, from a fallen stump, etc.), as well as notes on the number of individual tracks seen in the snow. In Session 3 the sampling effort for each field day per ranger team was also recorded. Every time we left the car and started a daily transect, the effort was recorded as GPS track in the app. The recording of the transect ended upon return to the car. This allowed us to estimate population parameters in a SECR modelling approach using the sampling effort as a model covariate (see below).

Collected hair samples from the field were shipped to the genetic laboratory on a monthly basis to avoid DNA degradation, and DNA extraction was performed within a week of a sample's arrival to the laboratory. We stored scat and urine at -20°C until shipped to the genetic laboratory at the end of each sampling session.

3.2. Genetic analyses

We genotyped the samples 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 used the Amelogenin locus for sex determination. Strict regimes were implemented for sample handling and analysis to avoid contamination, including a dedicated laboratory for pre-PCR handling of noninvasive genetic samples with strict contamination prevention protocols (Skrbinšek et al., 2010). DNA was extracted with the MagMAX Multisample Kit (ThermoFisher Scientific) using a liquid-handling robot (Hamilton STARlet) to increase throughput and decrease the possibility of sample handling errors. To ensure reliable genotyping of noninvasive samples, we used a modified multi-tube approach (Taberlet et al., 1996, Skrbinšek et al. 2010). In the first screening process, each sample was amplified with PCR and analyzed on an automatic sequencer (Applied Biosystem ABI 3130xl Genetic Analyzer) in two parallels. Samples that provided no specific PCR products at that stage were discarded; the others were analyzed up to eight times. After each genotyping run, we checked genotype reliability with the Reliotype maximum-likelihood approach (Miller, Joyce & Waits, 2002) and calculated the quality index (Miquel et al., 2006) for each sample.

To identify which samples have the same genotype (and should belong to the same individual), we used a slightly modified procedure described by Skrbinšek et al. (2020). Since we used a large number of microsatellite markers relative to the expected number of animals in the study area, we allowed for some mismatches between samples. We allowed for one mismatch that could be caused by allelic dropout to still

consider a match reliable, but didn't allow any mismatches that could be caused by false alleles (allelic incompatibilities between genotypes). To avoid possible problems with false alleles, we set a minimum threshold of two clear observations of an allele in separate analyses before the allele was considered 'true'. We considered any matches with up to 3 possible allelic dropouts and 1 possible false allele mismatches as a "possible match" and collected further evidence (additional repeats). If a genotype was reliably matched to another reliably genotyped sample, it was accepted even if the genotype reliability was below 0.98 threshold (since the probability of a reliable match to a reliably genotyped sample in presence of errors is marginal). If a sample was not matched to another reliable sample, the analysis was repeated until reliability reached the 0.98 threshold or discarded after 8 replications if this threshold was not reached. When the quality index of a sample was below 0.4, the unmatched samples were discarded regardless since we considered the DNA quality as too low to provide a reliable genotype. If there was any doubt in reliability of a genotype or its match with another reliably genotyped sample, analyses were repeated.

3.3. Wolf-dog hybridization, parentage analysis and field observations

To assess wolf-dog hybridization we used genotypes of 21 domestic dogs as a reference. We collected our reference dog samples during the same period and within the same sampling area as the wolf samples, to determine if the canids detected in the wild are pure wolves. We used Bayesian clustering in program STRUCTURE v.2.3.4 to detect hybrids (Pritchard, Stephens & Donnelly, 2000). Structure was run with 10^5 iterations of burn-in followed by 106 MCMC iteration. We used the population admixture model with correlated frequencies, used wild-collected genotypes and reference dogs in the same run. We explored K = 2, and used CLUMPAK (Kopelman et al., 2015) to interpret results from independent runs.

In parallel with sampling wolves in the wild, we sampled dogs in the villages within the project area. Dog owners helped us swabbing for saliva a total of 21 large-sized dogs. We used these samples as a reference for comparing the genetic structure of domestic and wild canids and eventually identify potential wolf-dog hybrids in the wild. Hybridization between wolves and dogs is a real threat on wolf population viability across Europe, especially in countries with human dominated landscape and fragmented wolf populations. Little is known about the magnitude of wolf-dog hybridization in Romania. Although field professionals reported direct observations of animals with wolf-dog morphological characteristics, little efforts were done for systematically assessing the problem.

Parentage and sibship assignments using genetic data enable us to identify family groups and estimate the number of packs present in the study area even when the data are too sparse to allow for a reliable capture-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 and sibship assignments in the same model, providing more efficient use of available data. We allowed for a locus-specific probability of allelic dropout error on each locus (between 0.017 and 0.073) and 0.004 probability of a false allele. We performed 3 independent runs using full-likelihood,

medium precision and long run. While wolves are typically monogamous, they can produce offspring with different partners during their lifetimes because of relatively high mortality (Skrbinšek, unpublished). While we explored both options, we made the final analysis with a model that assumes polygamy.

Wolves are social territorial animals, living in family groups (packs) or pairs, and are usually monogamous with only one breeding pair in the social unit (Mech & Boitani, 2003). In most cases, a pack also includes also the offspring of the mated pair, all of which eventually disperse, but may be accompanied, at least temporarily, by wolves unrelated to the pack. The parentage analysis assigned the related animals to a pack, but field observations suggested that there were genetically unrelated individuals travelling with the pack in some instances. Therefore, the results of pedigree reconstructions were complemented based on field observations on the number of distinct tracks visible in the snow. During the sample collection, we wrote notes regarding the number of individuals visible on the snow tracks. Furthermore, we also gained field observations about approximate pack size from intensive camera trapping performed with the purpose of monitoring Eurasian lynx in the same time interval and the same study area (Iosif et al., 2020, 2022). In a multi-generation parentage analyses, the limited sampling (inability to include all parents), the limited power of the marker panel and and possible genotyping errors may cause circular references to occur (i.e., an offspring from a later generation was identified also as parent in a previous generation). In these cases, we corrected the pack and status assignments based on field observations.

3.4. Population estimates

3.4.1 Non-spatial capture recapture models

Non-spatial capture recapture models (CMR) require encounter histories of the individuals in the study population (one capture/mark and subsequent recapture events) for estimating population size. While data from all sampling sessions was useful for understanding parental relations and packs dynamic in time, for CMR we used only the $S1_{sa}$ and $S3_{sa}$ for comparability (small area), and $S3_{la}$ (large area) dataset. We did not use the data from $S2_{sa}$ because the low sampling in that session did not provide enough recaptures for reliable estimate. Prior to summarizing the number of recaptures, we removed the spatially and temporally autocorrelated samples (samples of an individual collected less than 1 km apart on the same day) to avoid pseudoreplication.

To estimate abundance, we used five CMR modelling approaches that all assume a demographically closed population. We ran Chao's Mh and Darroch models (Chao, 1987), using R-package RCapture (Baillargeon & Rivest, 2007) which are relatively robust to capture heterogeneity of the individuals, and used AIC as the basis for model selection. We also ran two different models from the Capwire approach (Miller, Joyce & Waits, 2005) using the R-package Capwire (Pennell et al., 2013): 1) ECM model: all individuals are assumed to have an equal probability of being sampled (i.e., captured) on each sampling occasion. 2) TIRM model: the equal capture probability can be relaxed by allowing individuals to come from different rate classes: some individuals are easily captured while others are difficult to capture. For Capwire model selection we considered the likelihood ratio tests included in the Capwire R package. We ran the models for all individuals together and separately for males and females. In the final model selection, we also considered how consistently models performed between sampling sessions with the inherent assumption that drastic changes in abundance were less likely than abundance remaining around similar values between consecutive sessions.

3.4.2 Spatially explicit capture recapture models

The spatially explicit capture recapture (SECR) models are used to estimate the density of wildlife populations using individuals' encounters at a spatial detector array within the effective sampling area, addressing in this way the uncertainty related to the edge effect (Figure 1). The effective sampling area is the extension of the detector array, used to minimize the probability of detecting a wolf from outside of the study area during the sampling (Royle et al., 2014). We created the effective sampling area by adding a 2.5×σ (spatial scaling parameter; Supplementary Material 1) to the detector grid, i.e., getting the minimum X and Y coordinate of the detector array and subtract 2.5×σ from them, and getting the maximum X and Y coordinate of the detector array and add 2.5×σ to them.

We used the data from S3la session for SECR modeling. We generated the detector array as follows: 1) we divided the sampling area into 2.5×2.5 km grid cells (Supplementary Material 1) and counted the number of samples of each individual within each grid cell from November 2019 to May 2020. 2) The centroids of cells in which contained genotyped samples were named as detectors within the SECR modelling. Thereby, we got a dataset with the number of individuals' encounter at each detector (Supplementary Material 1). We also added the sampling effort to the model as detector level covariate. The sampling effort is the transect length within a 2.5×2.5 km grid cell (km km-2).

In this study we used the Bayesian hierarchical Markov Chain Monte Carlo (MCMC) model approach based on López-Bao et al., (2018) example. We ran three MCMC chains with 1000 burn-in steps and 50,000 iterations with thinning rate 5, resulting in 30,000 outputs. For checking the convergence of the MCMC chains we used the Gelman-Rubin statistic R-hat (Gelman & Rubin, 1992), where values below 1.1 indicated good convergence (Supplementary Material 1). The goodness-of-fit of models were tested threefold using Bayesian p-values described in (Royle et al., 2014): i). individual encounter frequencies per detector, ii). individual encounter frequencies aggregated for each individual; and iii) detector frequencies aggregated for each detector (Supplementary Material 1).

Chapter 4
Results

4.1. Genotyping success

During the three sampling sessions we collected 505 samples, 153 in Session 1, 99 in Session 2, and 251 in Session 3. Although the sampling window was open from 1 July to 31 June the following year, the systematic sampling started in November and ended in May. The samples collected between November and May represent 90% of the total samples. The remaining 10% we opportunistically collected in the summer season (Supplementary material 2).

We collected six types of non-invasive samples: scat and urine were the most frequent, followed by hair samples, while non-invasive blood (i.e., collected from snow), saliva and tissue were the rarest (Table 1). From the total amount of samples collected, 53% provided a reliable genotype (genotyped samples; Table 1). Samples with insufficient quality to provide a useful genotype (poor samples) represent 34%. DNA of two or more individuals (two wolves, wolf + fox, etc.) collected in the same sample (mixed samples) represent 5.6% of the total samples, while 7.5% of the samples contained DNA from other species, hereafter referred as non-target samples (Table 1). Urine samples had a higher amplification success than scat or hair samples (Table 1).

The mean field-estimated age of scat samples was 2 days (SE = 0.23) in Session 1, 1.8 days (SE = 0.21) in Session 2, while in Session 3 the mean scat age was 2.7 (SE = 0.15) (Supplementary material 2).

Table 1. Genotyping success by sample type across the three sampling sessions. Genotyped samples refer to reliable wolf genotypes, mixed refers to DNA from two or more individuals collected, Poor refers to samples with insufficient quality, and non-target samples refer to DNA from other species.

Sample type	Number of samples	Genotyped	Mixed	Poor	Non-target
Scat	354	180	10	133	31
Urine	123	76	16	26	5
Hair	20	6	2	10	2
Blood-noninvasive	4	3		1	
Saliva	2			2	
Tissue	2	1		1	
Total	505	52.7%	5.6%	34.3%	7.5%

4.2. Population dynamic and wolf pack structures

Across the three sampling sessions, we identified 48 individuals: 27 males and 20 females plus one F1 wolf-dog hybrid male (Figure 2). In $S1_{sa}$, we detected 25 individuals. The $S2_{sa}$ samples detected 7 new individuals but missed 14 of the original individuals detected in $S1_{sa}$. In $S3_{la}$ a total of 31 individuals were detected: 15 new individuals, 14 from $S1_{sa}$ and $S2_{sa}$, and the 2 individuals missed in $S2_{sa}$ but captured again in $S3_{sa}$ (Figure 2). The mean recapture rate for males was 6.3 (SE = 1.2) and 4.75 for females (SE = 0.92). The average recapture rate of reproductive individuals was 9 (SE = 2.28) while for offspring was 4.5 (SE = 0.64). In $S3_{sa}$, a higher number of individuals were detected due to the larger sampling area (see Materials and methods; Figure 2).
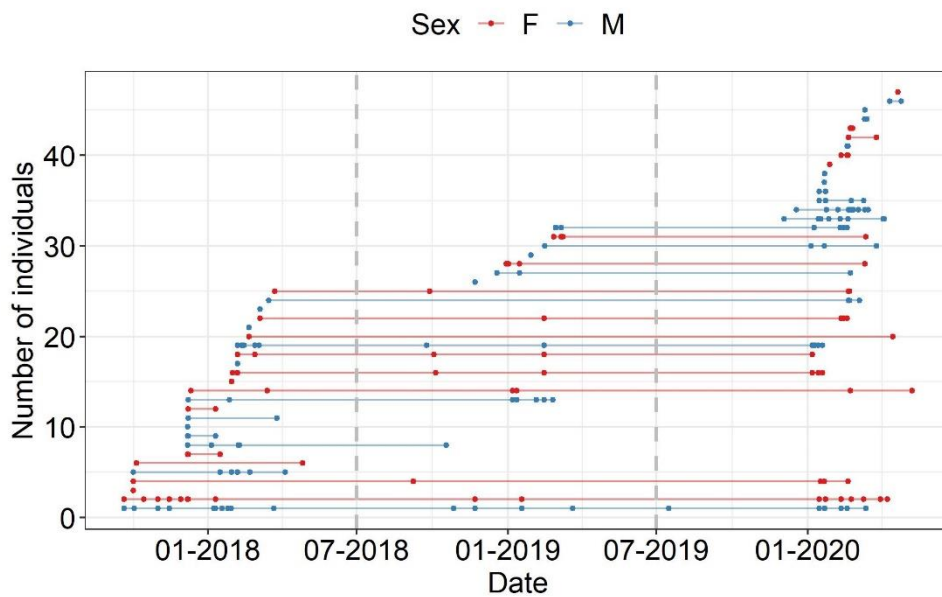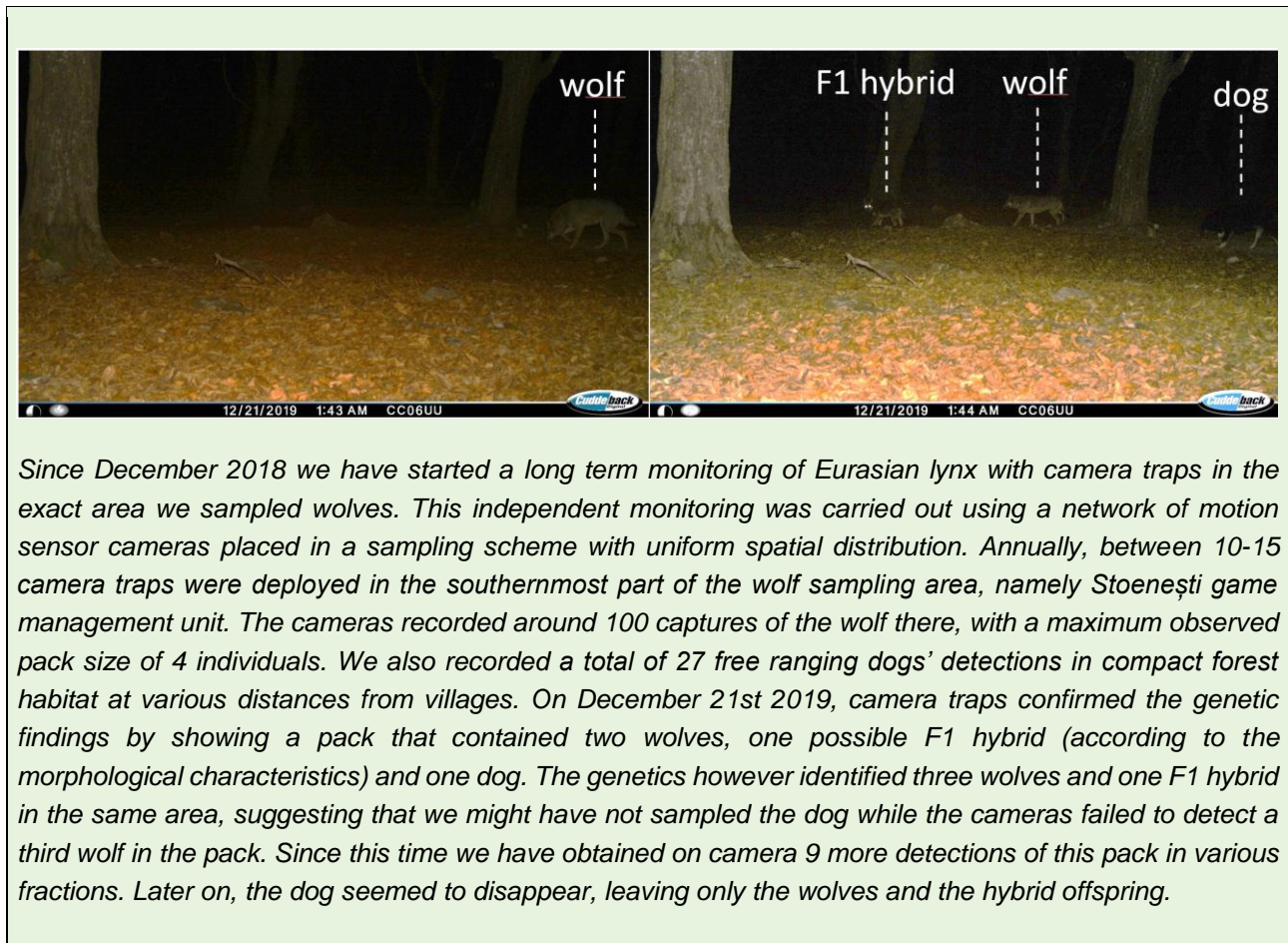

Figure 2. Recapture history of each individual wolf across the three sampling sessions. Sampling sessions are divided by the vertical dashed lines.

Structure software results show that of the 48 identified individuals, one was an F1 hybrid between wolf and dog (Supplementary material 3). Field observations and an independent camera trapping dataset also confirmed the presence of an animal with morphological characteristics of a wolf-dog hybrid, as well as a domestic dog accepted in a pack with two animals exhibiting typical wolf morphology.

Since December 2018 we have started a long term monitoring of Eurasian lynx with camera traps in the exact area we sampled wolves. This independent monitoring was carried out using a network of motion sensor cameras placed in a sampling scheme with uniform spatial distribution. Annually, between 10-15 camera traps were deployed in the southernmost part of the wolf sampling area, namely Stoeneşti game management unit. The cameras recorded around 100 captures of the wolf there, with a maximum observed pack size of 4 individuals. We also recorded a total of 27 free ranging dogs' detections in compact forest habitat at various distances from villages. On December 21st 2019, camera traps confirmed the genetic findings by showing a pack that contained two wolves, one possible F1 hybrid (according to the morphological characteristics) and one dog. The genetics however identified three wolves and one F1 hybrid in the same area, suggesting that we might have not sampled the dog while the cameras failed to detect a third wolf in the pack. Since this time we have obtained on camera 9 more detections of this pack in various fractions. Later on, the dog seemed to disappear, leaving only the wolves and the hybrid offspring.

After complementing the pedigree reconstruction with field observations, we grouped 40 wolves and the F1 hybrid into six packs (Supplementary material 4, Figures 3-9). The remaining 7 wolves could not be assigned to any specific pack and were assumed to be either dispersing animals or members of packs neighbouring the study area stochastically detected at the edge or outside of their territory. Except for one pack in the central part of the sampling area, individuals that were not genetically related to the pack were assigned to a particular pack as they move together or were detected in the approximate pack territories (Figures 4-9). Across all three seasons, we detected turnovers in the breeding pairs in 2 out of 6 packs (Figures 4-9). The detected number of animals per pack was between 4 and 7 in Session 1, between 2 and 6 in Session 2, and between 3 and 6 in Session 3 (Figures 4-9).
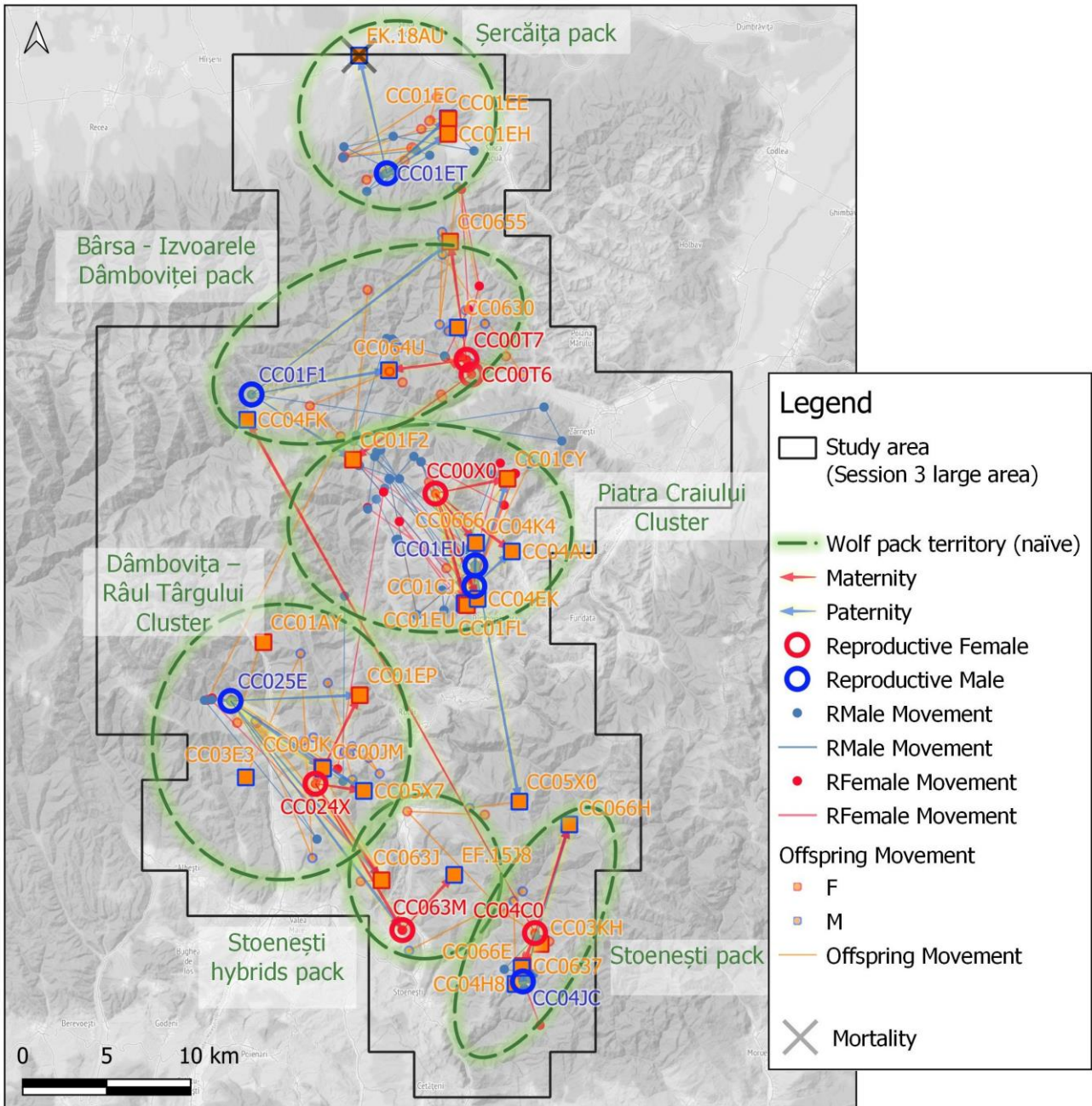
Figure 3. Distribution of wolf packs detected during three sampling sessions. The six packs contain 40 wolves and one F1 wolf-dog hybrid.

4.2.1. Piatra Craiului pack

Piatra Craiului pack during the first sampling session contained seven individuals: two reproductive individuals and five offspring. During Session 2 the pack split in two when CC01EU male became reproductive with an unknown female. In Session 3 there were three offspring: two from the original reproductive pair and one from CC01EU and an undetected female. The offspring of the second reproductive pair was already detected exploring new territories in the south, outside of the initial pack territory (Figure 4).
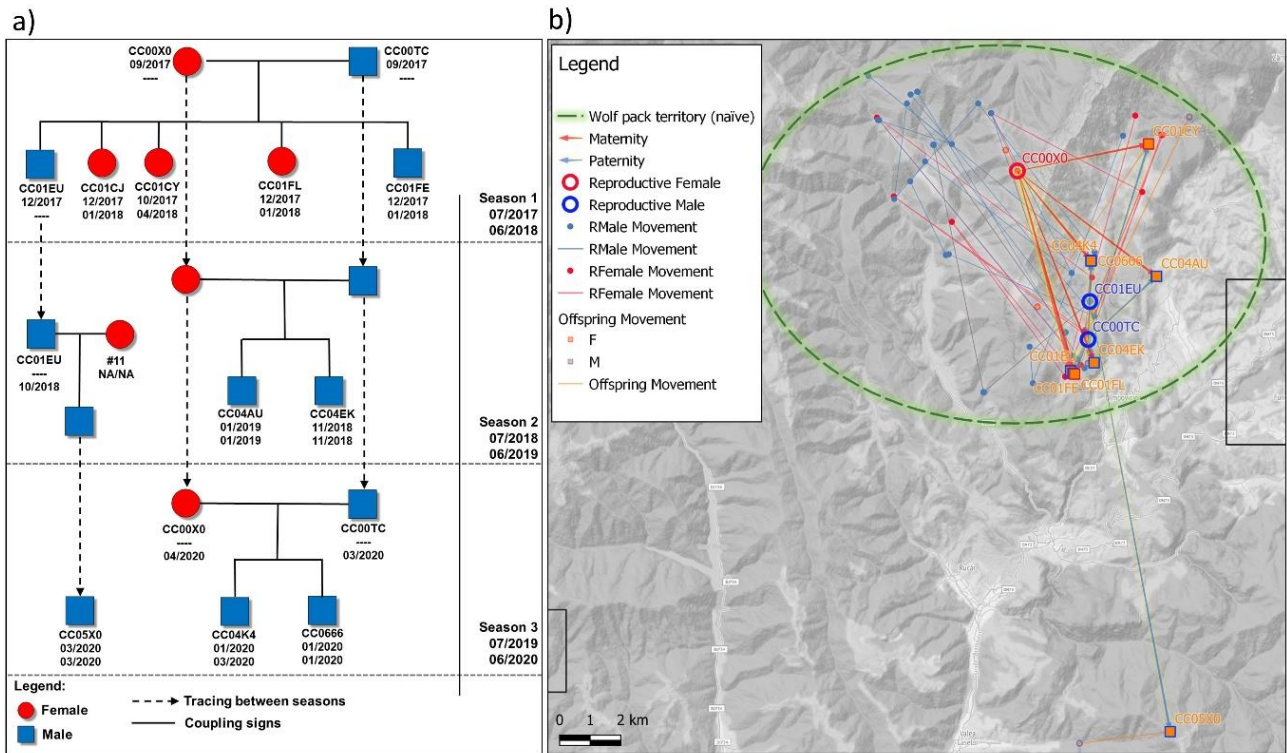
Figure 4. Panel a) Pack structure of Piatra Craiului pack representing the genetic relationship between individuals, their IDs, sex, date of first and last sample collected, the coupling signs and tracing over sampling sessions. The #11 indicates an undetected female. Panel b) map of the pack.

4.2.2. Bârsa – Izvoarele Dâmboviței pack

Bârsa – Izvoarele Dâmboviței pack during Session 1 contained four genetically identified individuals. The CC00T6 reproductive female had two offspring (CC01F2 and CC00T7) from an undetected male. CC01F1 male travelled with the pack although not genetically related. In Session 3, CC01F1 male became a reproductive and had two offspring with an offspring female CC00T7 from Session 1. CC01F2 offspring female from the Session 1 remained with the pack in the Session 3 as well. While CC01F1 male and CC00T7 female might have been reproductive pair in Session 2 as well, we got no samples from this area during that session. CC01F1 made circular reference from Session 1 to Session 3. This happens in multi-generation pedigrees with limited sampling and some genotyping errors. A male (CC0630) was assigned to pack in Session 3, but we didn't find genetic relationship between these individuals and other members of the pack (Figure 5).
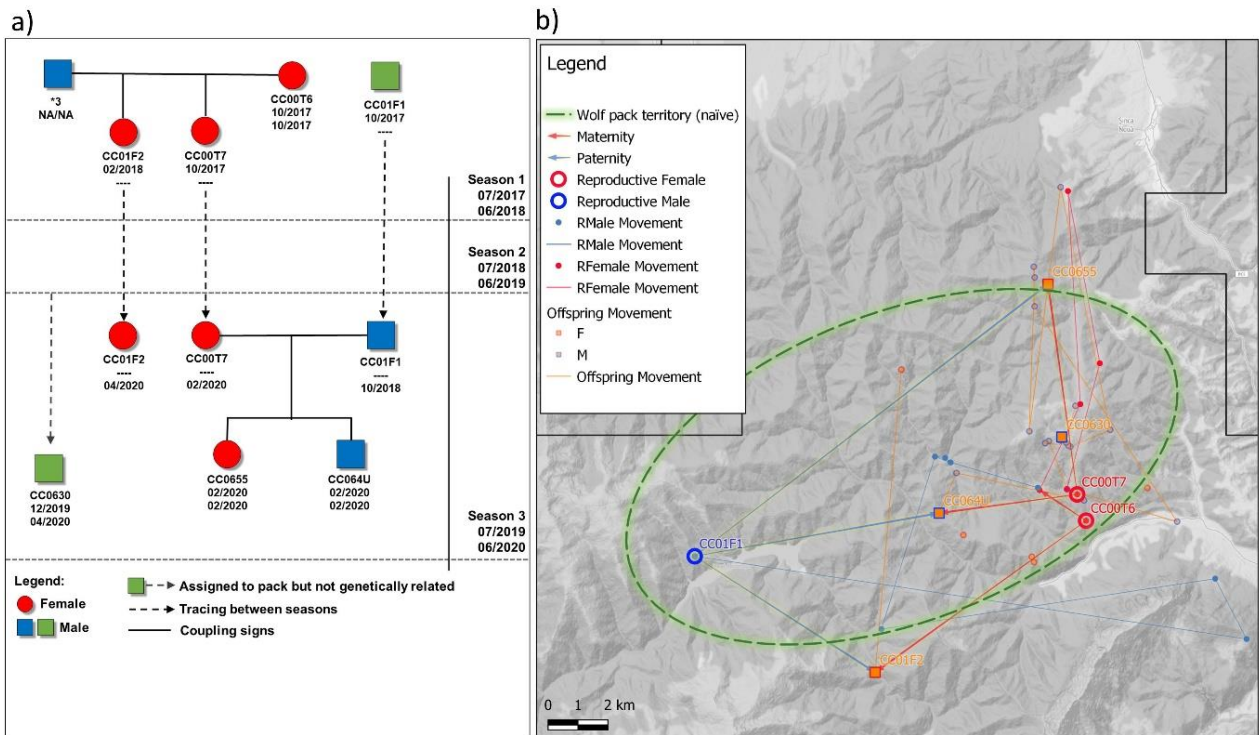

*Figure 5. Panel a) Pack structure of Bârsa – Izvoarele Dâmboviței representing the genetic relationship between individuals, their IDs, sex, date of first and last sample collected, the coupling signs and tracing over sampling sessions. Individual marked with green square means a male assigned to pack, they travel together but we didn't find genetic relationship. *3 marks an undetected male. Panel b) map of the pack.*

4.2.3. Șercăița pack

Initially, Șercăița pack had four identified individuals with genetic relationship between them and a female assigned to the pack but not genetically related. During the Session 2 and Session 3 we recaptured the reproductive male (CC01ET), an offspring female (CC01EH) and the assigned female (CC01EE) (Figure 6).
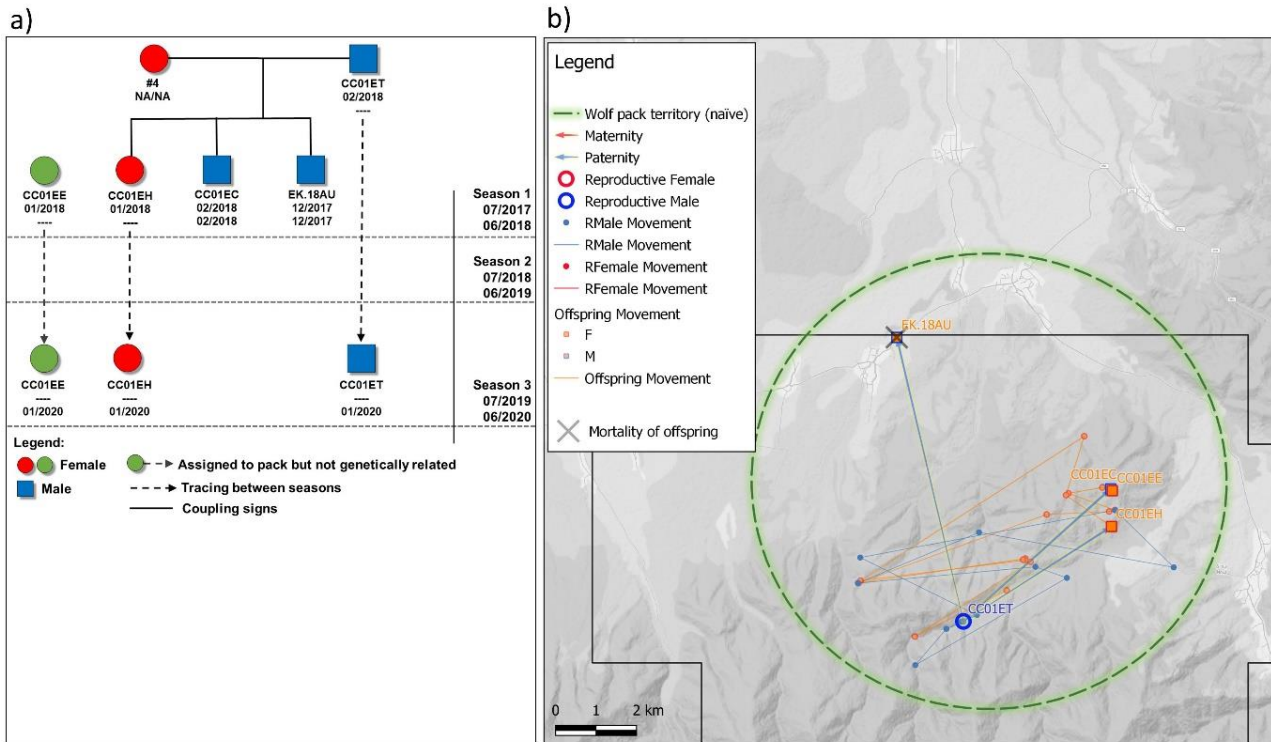

Figure 6. Panel a) Pack structure of Șercăița representing the genetic relationship between individuals, their IDs, sex, date of first and last sample collected, the coupling signs and tracing over sampling sessions. Individual marked with green circle represents a female assigned to pack, they travel together but we didn't find genetic relationship. #4 represent an undetected reproductive female. Panel b) map of the pack.

4.2.4. Dâmbovița – Râul Târgului pack

In Session 1, Dâmbovița – Râul Târgului pack contained a reproductive pair, their three offspring and an unrelated female CC01AY travelling with the pack. In Session 2, this reproductive pair had two offspring: CC063J and CC05X7, kept offspring CC00JK from Session 1 but skipped CC01EP and CC00JM. The pack in Session 3 contained the reproductive female from Session 1 and 2 (CC024X), the two offspring from Session 2 (CC063J and CC05X7), a female offspring from Session 1, CC00JK, and two genetically unrelated individuals CC03E3, CC01AY (Figure 7).
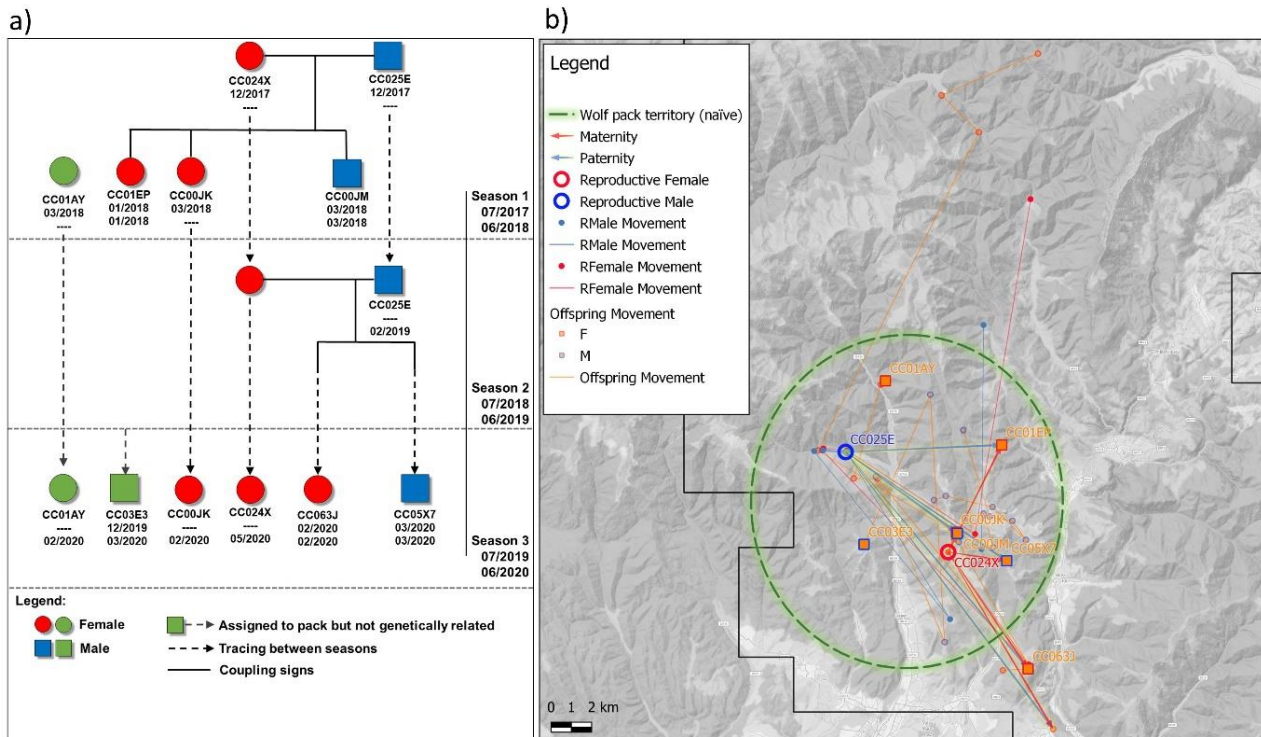

Figure 7. Panel a) Dâmbovița – Râul Târgului pack structure representing the genetic relationship between individuals, their IDs, sex, date of first and last sample collected, the coupling signs and tracing over sampling sessions. Individual marked with green circle represents a female, while green squares represent males assigned to pack, they travel together but we didn't find genetic relationship. Panel b) map of the pack.

4.2.5. Stoeneşti hybrids pack

Stoeneşti hybrids pack was first captured in Session 2, when included two pure yet unrelated wolves: male CC04H8 and female CC03KH. During Session 3, together with the male and female wolves detected in Session 1, we identified an F1 hybrid which was genetically related to a second wolf female CC063M and an undetected male (Figure 8).
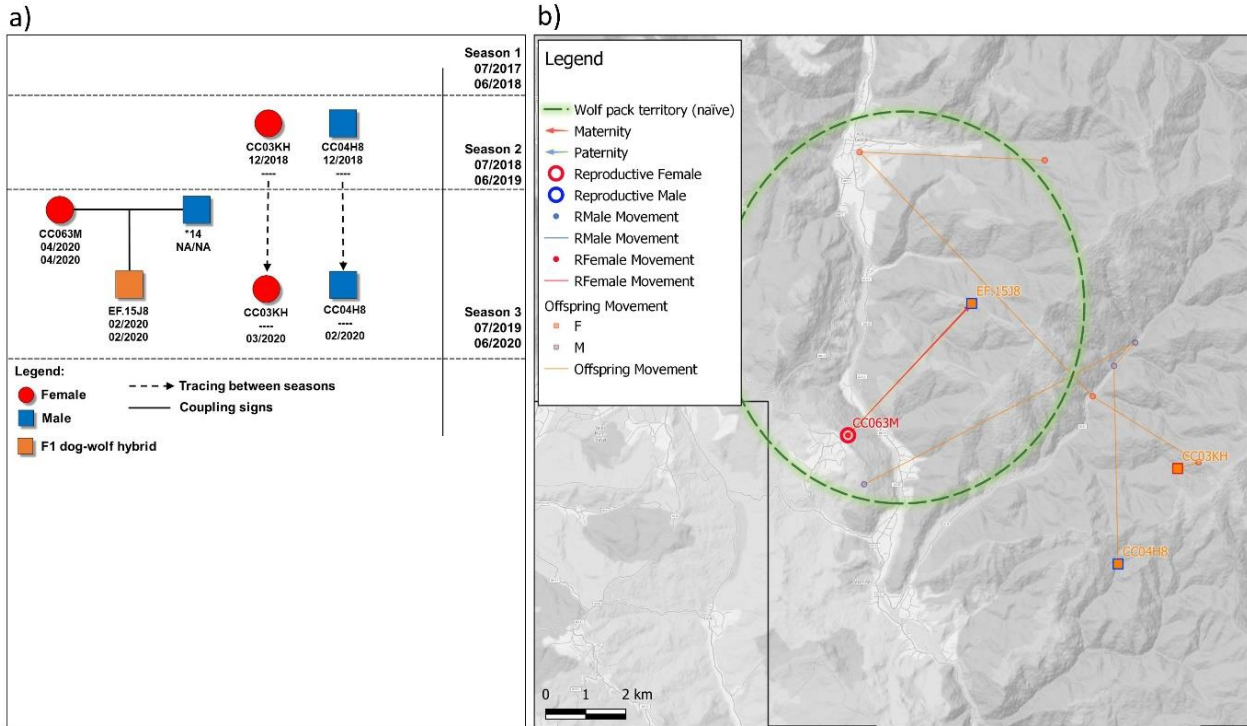

Figure 8. Panel a) Stoeneşti hybrids pack structure representing the genetic relationship between individuals, their IDs, sex, date of first and last sample collected, the coupling signs and tracing over sampling sessions. Individual marked with brown square represents an F1 dog-wolf hybrid. #14 represent an undetected male. Panel b) map of the pack.

4.2.5. Stoenești pack

Stoenești pack was first identified in Session 2: a reproductive male and a polygamous female (captured in Session 3). The reproductive female had an offspring male from an undetected male. Across sessions, this offspring male CC04FK left the pack and dispersed north to territories of Dâmbovița – Râul Târgului and Bârsa – Izvoarele Dâmboviței packs, where he seems to remain a solitary individual. In Session 3 the reproductive female had three offspring (a female and two males) with the reproductive male identified in Session 1 (CC04JC) (Figure 9).
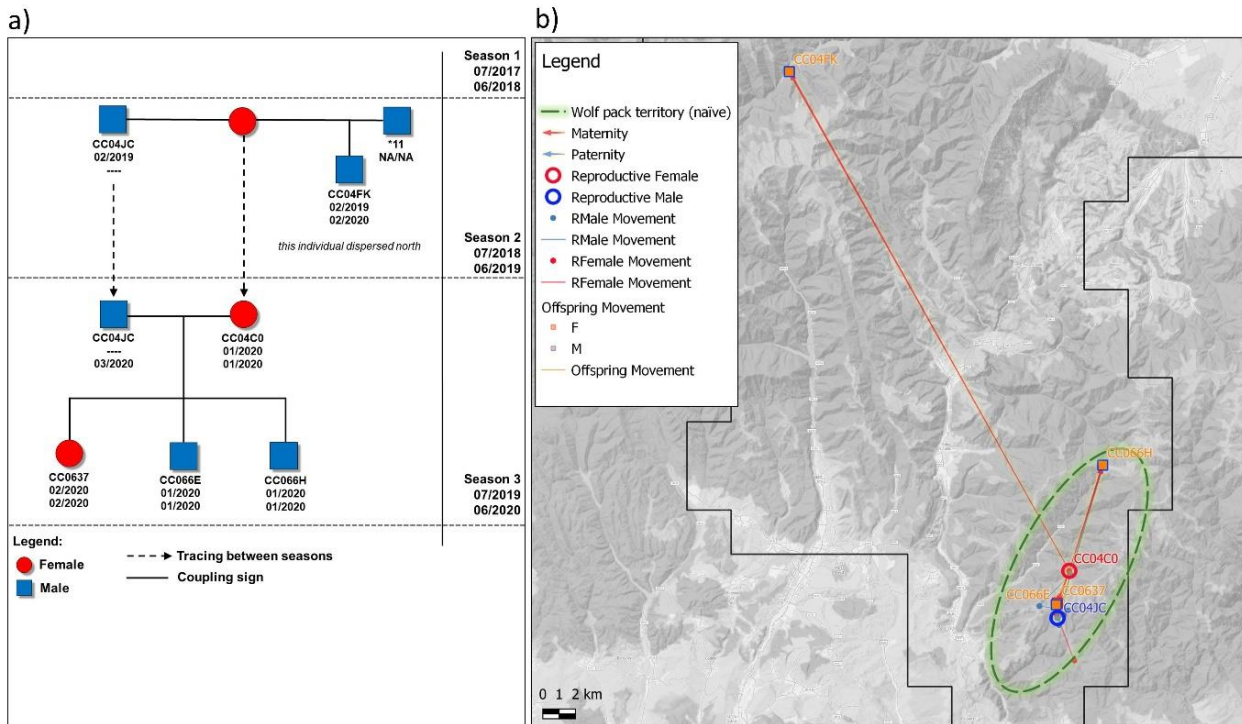

Figure 9. Panel a) Stoenești pack structure representing the genetic relationship between individuals, their IDs, sex, date of first and last sample collected, the coupling signs and tracing over sampling sessions. CC04FK male dispersed north. *11 marks an undetected female. Panel b) map of the pack.

4.3. Demographic estimates

The $S1_{sa}$ dataset contained 20 individuals (11 females and 9 males), the $S3_{sa}$ contained 19 individuals (10 females and 9 males), while in $S3_{la}$ the dataset contained 28 individuals (14 females and 14 males). From the different CMR models, we selected Chao's Mh model for population size estimates and comparison across sampling sessions because it assumes capture heterogeneity had better fit compared to other models and performed more robustly over sampling sessions (Figure 3; Supplementary Material S1).

The estimated population size in $S1_{sa}$ was 25 individuals (95%CI = 16-34), 9 males (6-13) and 16 females (8-25), with all models showing a similarly skewed sex ratio (Figure 10; Supplementary Material 1). Higher sampling effort distributed in $S3_{la}$, increased the robustness of the prediction on the $S3_{la}$ but decreased it in the $S3_{sa}$. In $S3_{la}$, the estimated population size was 31 (25-38), number of males was 16 (10-22) and the number of females was 17 (12-22) (Figure 10). Therefore, we estimated a balanced sex ratio of 1.06 (0.57-2.2). In $S3_{sa}$, lower number of captures affect the estimates (especially for males): estimated population size

was 21 (15-27), estimated number of males was 9 (8-10) and number of females was 14 (4-24) (Figure 10), driving the sex ratio from 2.11 (1.07-5.66) to 1.55 (0.4-3).
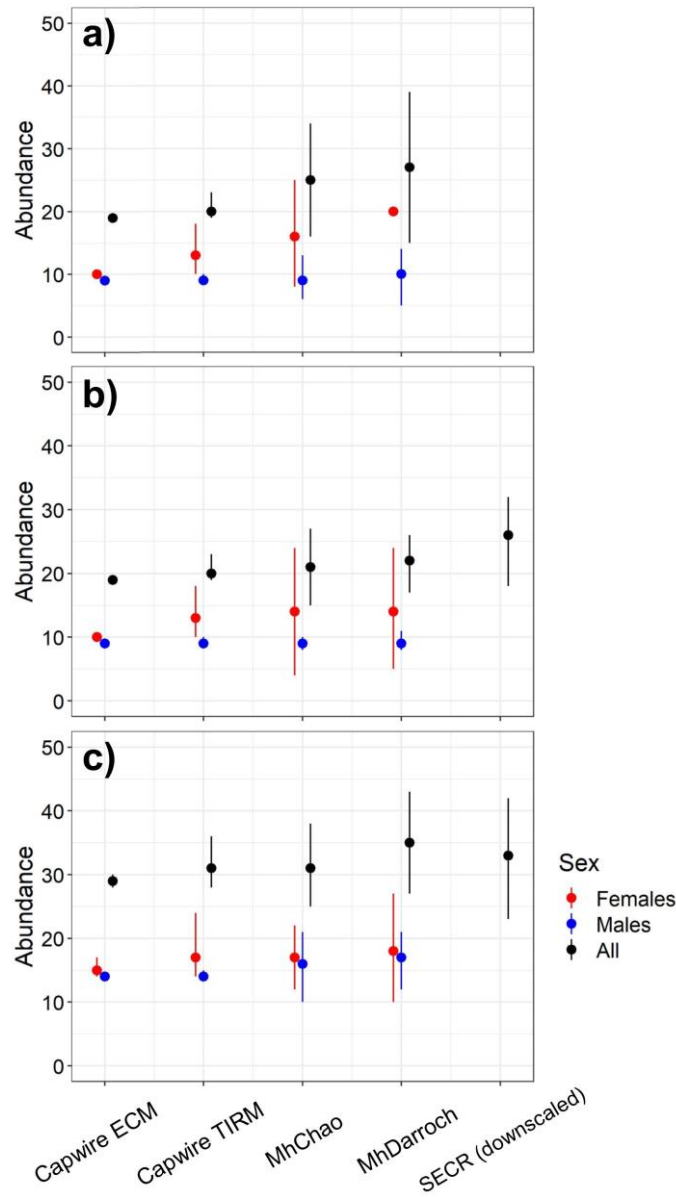

Figure 10. Non-spatial capture recapture models were used to estimate the regional abundance of wolves. Panel a) shows comparison between different models in S1$_{sa}$, panel b) in S3$_{sa}$ and panel c) in S3$_{la}$. ECM and TIRM refer to Capwire models in the R-package Capwire. We also used robust models that include capture heterogeneity such as Chao's Mh model and Darroch implemented in R package Rcapture. Note that in S3 we also plotted the abundance of all individuals independent on their sex as resulted from a spatially explicit capture recapture model and downscaled from the effective sampling area of the spatial model to S3$_{sa}$ and S3$_{la}$ of the non-spatial models.

Within the SECR framework, after applying an approx. 10 km buffer (> 2.5 × σ) around detectors we obtained an effective sampling area of 2975 km^2, which is 2 times larger than the S3$_{la}$ used in CMR. Thus, the population size estimates with SECR refer to a regional population size and is not comparable with CMR estimates as they refer to different effective sampling areas (Supplementary Material S1). The posterior mean population size, $\hat{N}$ was 70 individuals in SECR (95% Bayesian Credible Interval; BCI = 50-90). When downscaling population size obtained with SECR to the S3$_{sa}$ and S3$_{la}$ we obtained similar prediction as in the case of CMR (Figure 3). On the SECR effective sampling area, the posterior density estimate ($\hat{D}$) was 2.35 wolves/100 km^2 (BCI=1.68–3.03). The posterior estimate for σ parameter was 0.33 (95%BCI = 0.28-0.38; Supplementary

Material 3). The posterior mean density of activity centers of wolves across the effective sampling area is presented in Figure 11.
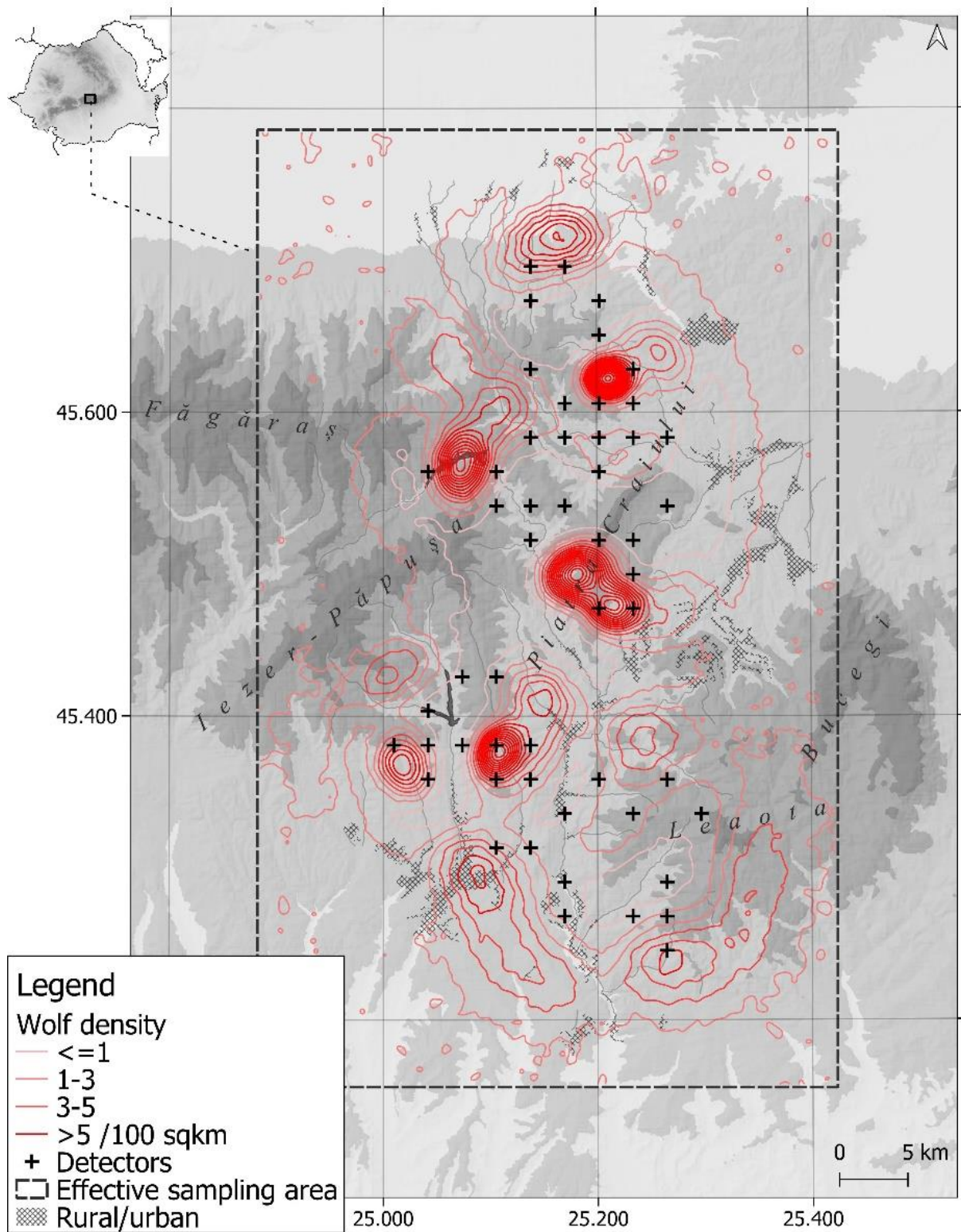

Figure 11. Posterior mean density of wolf activity centers reported for 100 km². The detector locations used for interpolating density surface are plotted on the map. The effective sampling area, 2975 km², is obtained by buffering the σ spatial scaling parameter 2.5 times around the detectors (see Methods).

Chapter 5
Discussion

We provide an assessment of wolf population parameters such as density, population size and sex ratio, as well as an assessment of pack structure and dynamics over three years in one of Europe's strongholds for this species: Romanian Carpathians. We used non-invasive DNA sampling and both non-spatial and SECR models to analyze our wolf recapture histories and derive population estimates. We provide metrics essential for management and compare it with similar studies across the species range. These findings are contextualized to the local particularities that challenge wolf conservation and coexistence with livestock breeders and local hunters. Finally, we discuss implications for expanding this case study and eventually advance towards a transparent coexistence management based on scientific data across the Romanian Carpathians.

The estimated density of 2.35 wolves/100 km^2 (BCI=1.68–3.03) is lower than the density estimated in Northern Range, Yellowstone National Park, where it varied between 3.5 - 9.8 wolves/100 km^2 (Mech & Barber-Meyer, 2015). In central Italy, at the border of the Apennine chain where habitats are more fragmented than in our sampling area, the density - calculated as packs per 100 km^2 - was 1.3 packs, with an average pack size estimated at 4.2 wolves during winter (Mattioli et al., 2018). In the Northern Apennines, Apollonio et al., (2004) estimated a density of 4.7 wolves/100 km^2, two times larger than in our sampling area. In the Scandinavian population, in ecosystems driven by different climatic conditions, the average wolf density is significantly lower than in our sampling area: there are estimated to be 0.18 wolves/100 km^2, with just a few hotspots where

density goes up to 2 wolves/100 km^2 (Milleret et al., 2021). However, that particular population is still recovering from a recolonization bottleneck, so it may not be directly comparable.

With 1.2 wolves/100km^2, the estimated density in north-west Poland was lower than our results but had a comparable pack size range of 3.5 – 5.6 wolves (Mysłajek et al., 2018). In the Polish Carpathians, however, the density in early winter was two times higher at 5.1 wolves/100km^2, yet comparable with our sampling area in late winter with 3.3 wolves/100km^2 (Śmietana & Wajda, 1997). The Cantabrian wolf population in Spain was evaluated at similar density of 2.5 wolves/100 km^2 (López-Bao et al., 2018). In the Eastern Romanian Carpathians, in a similar environment and with a similar methodological approach, Gazzola et al., (2017) calculated a density ranging from 1 to 2.8 wolves/100 km^2 and with similar pack size (3 to 9) across a four-year survey. Among the factors regulating wolf density, the prey abundance and biomass have a major impact (Mech & Barber-Meyer, 2015; Serrouya et al., 2017). In the Romanian Carpathians the abundance of ungulate prey is considered to be lower than in other places across Europe, but this is based on expert opinion only as little quantifiable data is available on ungulates. Low abundance of the ungulate species in the Romanian Carpathians has been linked to corroborated pressure from carnivore predation and trophy hunting (Melis et al., 2013), low food availability at high altitudes (Geisser & Reyer, 2005), and more recently, infectious diseases (Morelle et al., 2020). Low prey availability may regulate the wolf population size at the peak of the trophic pyramid, resulting in lower densities than in other places across the species range. Another possible explanation for the differences in density is the intact community of large carnivores with high inter-specific co-occurrence in the Romanian Carpathians (Dyck et al., 2022), as opposed to other mountain ranges in Western and Southern Europe – particularly where other large carnivores have gone locally extinct. At 18 bears/100km^2 in the same sampling area and time frame (Iosif et al., 2021), the high density of brown bears in the Romanian Carpathians may act as a regulating factor for the density of wolves. Ordiz et al., (2015) demonstrated that Scandinavian wolf pair establishment was negatively correlated to bear density. Kleptoparasitizing behaviour has been documented for bears, where large amounts of competing apex predator kills are stolen. Among Eurasian lynx, 50% of prey kills were kleptoparasitized by bears in the temperate forest of the Dinaric Mountains (Krofel, Kos & Jerina, 2012).

The pack structure was highly dynamic from year to year. Not only were there three detected dispersers between known packs, we even had reproductive individuals disappeared from one sampling session to another. This led some reproductive individuals mating with different partners across sampling sessions. We detected turnovers of breeding pairs in 2 out of 6 packs. In three packs we detected at least one individual not genetically related with the other members of the pack, moving with pack members or randomly detected while dispersing within the approximate pack territories. On the one hand, this dynamic may indicate a good connectivity and gene flow between neighboring packs in our population. Ericson et al., (2020) found weak population differentiation in wolves across the Romanian Carpathians, the traditional agricultural mosaic offering connectivity between different mountain ranges especially in the absence of highways crossing the Carpathians. Similar inferences were made in the case of Eurasian lynx in the same area. Iosif et al., (2022) showed lynx density increases during the winter in the well-connected agricultural mosaic around the mountains. However, since wolves consume prey much faster than lynx, we can assume this effect to be less important for wolves than for lynx.

On the other hand, turnover in our wolf breeding pairs and variable pack sizes across sessions may indicate that different threats - including human-induced mortality - put pressure on our wolf population. Among other canids for example, the loss of the adult members in the African wild dogs due to human-induced mortality translated into food insecurity for the young members of the group (Courchamp & Macdonald, 2001). Ausband, Mitchell & Waits, (2017) found that breeder turnover in wolves resulted in changes to group composition, increased polygamy, further resulting into changes in the genetic content of a pack and impacting short-term

population growth. Since the trophy hunting ban in 2016, our Carpathian wolf population was not subject to harvesting, however different type of human-induced mortality, especially outside of the protected areas, remains unassessed. Wolf persecution from the livestock breeders as a response to predation of different livestock may be a possible explanation for pack dynamics (Santiago-Avila et al., 2018). In packs living at the transition between compact forest and the traditional agricultural mosaic, the illegal kills are documented to increase with habitat fragmentation across North America (Hill et al., 2022). Persecution through poisoning was documented in a similar coexistence landscape in Italy, but the spatial distribution of illegal wolf killings indicates that persecution cannot be blamed on a single category of persecutors such as the farmers (Musto et al., 2021). This suggests that the damage patterns have complex causes with social and institutional implications. Sin et al., (2019) showed that wolf diet in Eastern Romanian Carpathians is mostly comprised of wild ungulates. Wild boar is the most common prey, followed by roe and red deer. Domestic species were found to have marginal importance with dogs being predated more than sheep, goat and horse (Sin et al., 2019). While this study covered a compact forest habitat, future research should evaluate wolf predation on sheep in more fragmented landscapes such as the Southern Carpathians. The competition between wolves and hunters on harvesting ungulate prey can lead to the illegal killing of wolves. In Poland, six out of seven sentenced wolf poachers were hunters, responsible for illegal killings, including of breeder individuals, that verifiably impacted the wolf pack dynamic (Nowak et al., 2021). Illegal shooting of wolves in Romania outside of protected areas or during ungulate hunting remains only anecdotal. Unofficial evidence is emerging from the rural areas around the Romanian Carpathians revealing - similar to what was found in Sweden and Norway - a lack of transparency in reporting and addressing illegal shooting (Riener, 2019; Skogen, 2022). This reluctance is driven by frustration over official environmental policies with a strong perception of unjust power relations, not only among local hunter communities but across the entire rural community (Skogen, 2022).

Increasing road traffic in recent years with little institutional efforts to ensure safe passes for wildlife is another uncertainty that can lead to pack structure instabilities. Road kills are documented to have significant impact on wolf populations in Italy (Musto et al., 2021) and in the United States (Chakrabarti et al., 2022). We confirmed one case of a road-killed wolf during our sampling, but without a systematic sampling design along roads we could not assess the true magnitude of road mortality with our data.

Complementing the pedigree reconstruction with field observations helped us improving our inferences on pack composition but had its limitations as well. Errors can be induced when assigning one unrelated individual to a pack by looking at the number of tracks visible on the snow corroborated with the day and place we collected the samples. Wolf dispersers or individuals from neighbouring packs can also follow the same route and mark in the same place as the established pack with the sampler not being able to discriminate how many wolves travelled together, if the unrelated wolves were actually accepted in the pack, or just marking on top of each other. While the approach of complementing pedigree reconstruction with field observation may induce bias in the pack composition and pack size, it is not impacting on the population estimates.

We confirmed one of our sampled individuals was an F1 wolf-dog hybrid, to our knowledge the first genetic confirmation of a hybrid in the Romanian Carpathians. Field observations and an independent camera trapping study in the same area and time frame also showed one "hybrid-looking" animal moving together with a domestic dog and two "pure-looking" wolves. Although hybridization in our population appears marginal at this time, the independent camera trapping study reiterates that the magnitude of free ranging domestic dogs co-occurring in wolf habitat is a real concern (Iosif et al., 2020). Although a temporal avoidance in the activity of dogs and wolves is revealed, there is one free ranging dog encounter for every two wolf encounters in the wolf habitat in our studied population (Campbell, 2021). In human-dominated landscapes, human-induced habitat loss combined with higher interferences of free ranging domestic dogs degraded the genetic health of wolf populations, highlighting the need for maintaining large wolf populations to limit the introgression rates from

dog genetic material (Pilot et al., 2021). In the fragmented landscape of the Apennines for example, hybrids represented 4% of the sampled wolf population (Caniglia et al., 2014). In Galicia, NW Spain, hybrids represented 6% of the detected wolf population (Pacheco et al., 2017). More research is needed to understand wolf-dog hybridization in the Romanian Carpathians and to provide management solutions that limit dispersal of free ranging dogs.

Conservation and management implications: our results suggest that monitoring wolf population, pack structure, and dynamic as well as hybridization is feasible in the Romanian Carpathians by means of non-invasive DNA sampling during consecutive winter sessions. Genetic wolf monitoring can be expanded in several pilot areas representative for the extensive and diverse Romanian Carpathians (see Gazzola et al., 2017, for another pilot area in Eastern Romanian Carpathians). We showed that robust population estimates such as population size, number of packs, and local density can be easily obtained in a single winter sampling session and with a sampling effort of approx. 250 samples for our 1400 km^2 area. With more than 50% reliable genotypes, the density estimate was robust in a SECR modeling framework. However, strength, especially with respect to pack dynamics and hybridization, should be increased by continuous sampling over longer periods of up to 10 years and in pilot areas of 1000-2000 km^2 or larger. Such a monitoring scheme would improve the nation-wide wolf estimates and provide critical information on population dynamics for a wolf population that is only recently revealing its uncertainties through quantitative, scientific knowledge. We conclude that reliable estimates of population size, density, pack size and pack dynamics are essential information for achieving long term conservation and coexistence goals. This will help advance the closed decision-making processes to a transparent, coexistence-focused management strategy based on scientific data in the face of rapid environmental changes that affect the Carpathians, including habitat loss and fragmentation, climate change, and unpredictable wildlife management.

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Acknowledgements