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Estimation of population sizes in large carnivores in Europe using genetic analyses

BACHELOR'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled Estimation of population sizes in large carnivores in Europe using genetic analyses independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague date 16th April 2021

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Abstract

Large carnivores such as brown bear (*Ursus arctos*), Eurasian lynx (*Lynx lynx*) or grey wolf (*Canis lupus*) play very important roles as keystone species in their ecosystems. However, these species are particularly difficult to monitor through traditional field methods. Literature research was mainly focused on non-invasive genetic monitoring as a tool to estimate population sizes in large carnivores. This method was described along with its advantages and disadvantages and it was also compared to traditional field methods, which were the only source of data in the past. In non-invasive genetic monitoring, population sizes are usually estimated under Capture-Recapture modelling framework. This method and its estimation models (Closed population model, Open population model, Robust design) were characterised with examples of their applications in European countries. Practical part consisted of experimental estimation of wolf population size in the Czech Republic. Data set used to estimate population size contained 184 wolf samples which were collected between years 2014–2021 in the Czech Republic. Microsatellites were used as genetic markers and 156 unique genotypes were identified. Estimation of population sizes was carried out in software *capwire* using ECM and TIRM models. TIRM showed slightly higher likelihood, therefore we assume that this model is more suitable for estimating size of wolf population in the Czech Republic. However, our results were strongly overestimated possibly due to low number of samples used in our estimation, but also due to transboundary and geographic layout of wolves in the Czech Republic. Therefore, future efforts to estimate size of wolf population should take in consideration these factors causing possible bias in results.

Key words: carnivora, DNA analysis, genetic monitoring, microsatellites, non-invasive samples, population size

Abstrakt

Velké šelmy jako je medvěd hnědý (*Ursus arctos*), rys ostrovid (*Lynx lynx*) nebo vlk obecný (*Grey wolf*) hrají klíčové role v jejich ekosystémech. Nicméně monitoring těchto druhů pomocí tradičních metod je velice složitý. Literární rešerše byla zejména zaměřena na neinvazivní genetický monitoring jakožto nástroj k odhadování velikostí populací velkých šelem. Byly zde popsány výhody a nevýhody této metody a zároveň byl genetický monitoring porovnán i s tradičními metodami monitoringu, které byly v minulosti jediným zdrojem dat. Odhadování velikostí populací pomocí neinvazivního genetického monitoringu je většinou prováděno skrze modelový rámec metody Capture-Recapture. Tato metoda byla popsána společně s jejími modely (Closed population model, Open population model, Robust design) a příklady aplikací těchto modelů v Evropských zemích. Praktická část spočívala v pokusném vytvoření odhadu velikosti populace vlků v České republice. K tomu byl použit data set obsahující 184 vlčích vzorků, které byly nasbírány mezi roky 2014–2021 na území České republiky. K identifikaci jedinců byly použity mikrosatelity, pomocí kterých bylo identifikováno 156 unikátních genotypů. Vytvoření odhadu velikosti populace bylo provedeno v programu *capwire* s použitím modelů TIMR a ECM. TIRM vykazoval mírně vyšší statistickou věrohodnost, proto lze usuzovat, že by mohl být vhodnější pro odhadování velikostí vlčích populací v České republice. Naše výsledky byly nicméně silně nadhodnoceny, což bylo pravděpodobně způsobeno malým počtem použitých vzorků, ale i geografickým rozložením vlků v České republice. Tyto faktory, ovlivňující přesnost výsledků, by tudíž měly být zohledněny při budoucím odhadování velikostí populací.

Klíčová slova: analýza DNA, carnivora, genetický monitoring, mikrosatelity, neinvazivní vzorky, velikost populace

Contents

List of figures

List of tables

List of the abbreviations used in the thesis

AOPK – Agentura ochrany přírody a krajiny České Republiky

ATE buffer – Washing buffer

CJS model – Cormack-Jolly-Seber model

CMR – Capture-Mark-Recapture

CR – Capture-Recapture

DNA – Deoxyribonucleic acid

DTE buffer – Dithioerythritol buffer

ECM – Equal Capture Model

IUCN – International Union for Conservation of Nature

JS model – Jolly-Seber model

ME – Ministry of the environment

PCR – Polymerase chain reaction

SNP – Single nucleotide polymorphism

TIRM – Two-Innate Rates Model

1. Introduction

Large carnivores are among the most controversial and challenging species to conserve due to deeply rooted hostility against these species in human culture and history (Chapron et al. 2014). These species were once widespread across most of the European continent. Nonetheless, conflict between humans and large carnivores led to fragmentation of populations and their habitats and even extinction of large carnivores in some parts of Europe, particularly in Western Europe (Breitenmoser 1998; Marescot et al. 2011; Ripple et al. 2014; Boitanni & Linnel 2015).

After centuries of persecution and population decline, species of large carnivores such as brown bear (*Ursus arctos*), grey wolf (*Canis lupus*) and Eurasian lynx (*Lynx lynx*) are now expanding in Europe (Caniglia et al 2011). Unfortunately, return of large carnivores has brought back conflict between humans and large carnivores, especially in rural areas (Skogen, Mauz, Krange 2006). Therefore, it is crucial for management and conservation of these species to determine patterns and rates of population expansions.

Monitoring of large carnivores through standard field methods is challenging because they have strong tendency to avoid humans. Nonetheless, recent development of non-invasive genetic sampling and molecular identification of species, gender and individuals could be possible solution to reliable monitoring method of large carnivores (Caniglia et al 2011). Therefore, non-invasive sampling is increasingly used under a capture–recapture modelling framework to accurately estimate survival rates, capture probabilities, population trends and population sizes in large carnivore populations (Marucco et al. 2009).

2. Aims of the literature research

- To evaluate available data from genetic monitoring of large carnivores in Europe
- To create an estimations of population sizes in selected species
- To create an overview of methods used

3. Literature Review

3.1. Large carnivores in Europe

Carnivores play very important role in regulating ecosystems which people have not been able to replicate yet. They are often referred as a keystone species that can function as a flagship for conservation of the rest of the biodiversity. This means that conservation of large carnivores also conserves many other animal and plant species within their habitat (Linnel, Swenson, Andersen, 2000).

3.1.1. Species distribution

Populations of large carnivores have benefited from conservation laws shared by many countries in Europe, rise of environmentalist movements and from political stability through continent. These conditions made possible for pan-European legislative agreements to emerge which aim to protect biodiversity within European continent (Chapron et al. 2014). Legislative acts, reforestation, stabilization of large herbivore populations and positive public view towards large carnivore species in last 50 years have allowed their populations to grow (Epstein & López-Bao & Chapron, 2016; Chapron et al. 2014).

Currently there are four species of large carnivores inhabiting Europe, brown bear (*Ursus arctos),* grey wolf (*Canis lupus*), Eurasian lynx (*Lynx lynx*) and wolverine (*Gulo gulo*). All four species in Europe can be found outside of protected areas and even in human dominated landscapes. Together, these four species inhabit almost one third of total area of continental Europe with several stable populations distributed across continent (Boitani, Linnel, 2015; Chapron et al. 2014).

Wolves and bears have appeared to be very resilient towards human activities and they are able to live even within highly human-dominated European landscapes (Linnel et al. 2004). However certain small populations are still threatened, and their existence depends on conservation efforts. Growth of large carnivore populations in Europe and

their occurrence in human-dominated landscapes, have brought an increasing need for monitoring and management of these populations (Gese 2001; Linnel et al. 2004).

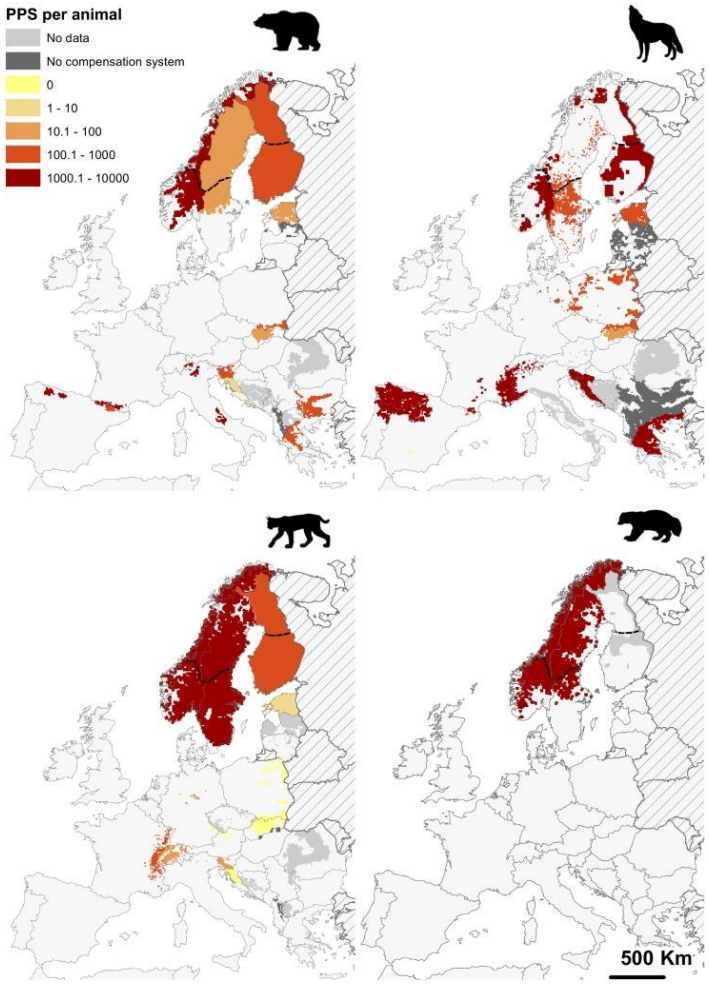

Figure 1: Distribution of large carnivore species across Europe (source: Bautista et al. 2019)

3.1.2. Ecological effects

Large carnivores with their position on the top of the food web have cascading influence on their ecological communities and ecosystems through their direct and indirect effects across lower trophic levels. Such effect is called "trophic cascade" and it makes large carnivores a crucial part of their ecosystems (Paine 1980; Beschta, Ripple, 2009). Pace et al. (1999) refined term trophic cascade as "reciprocal predator-prey effects that alter the abundance, biomass or productivity of a population community or trophic level across more than one link in the food web." This means that cascading influences

of large carnivores spread to other species through their influence on other species such as competition with species of mesocarnivores (e. g. foxes, coyotes) or predation on herbivores. Their impact variates, depending on many factors such as their hunting tactics, population density or their body size. Large carnivores do not only maintain carrying capacity of their ecosystems but indirectly influence their ecosystems in many ways. They maintain population sizes of their prey herbivore species, thus, allowing plants to grow and enhance carbon storage. They are also able to influence scavenger diversity which contributes to nutrient cycling (Schmitz et al. 2010; Wilmers et al. 2003). Large carnivores also help to reduce disease prevalence in prey population, thus, prevent spread of disease to livestock (Packer et al. 2003).

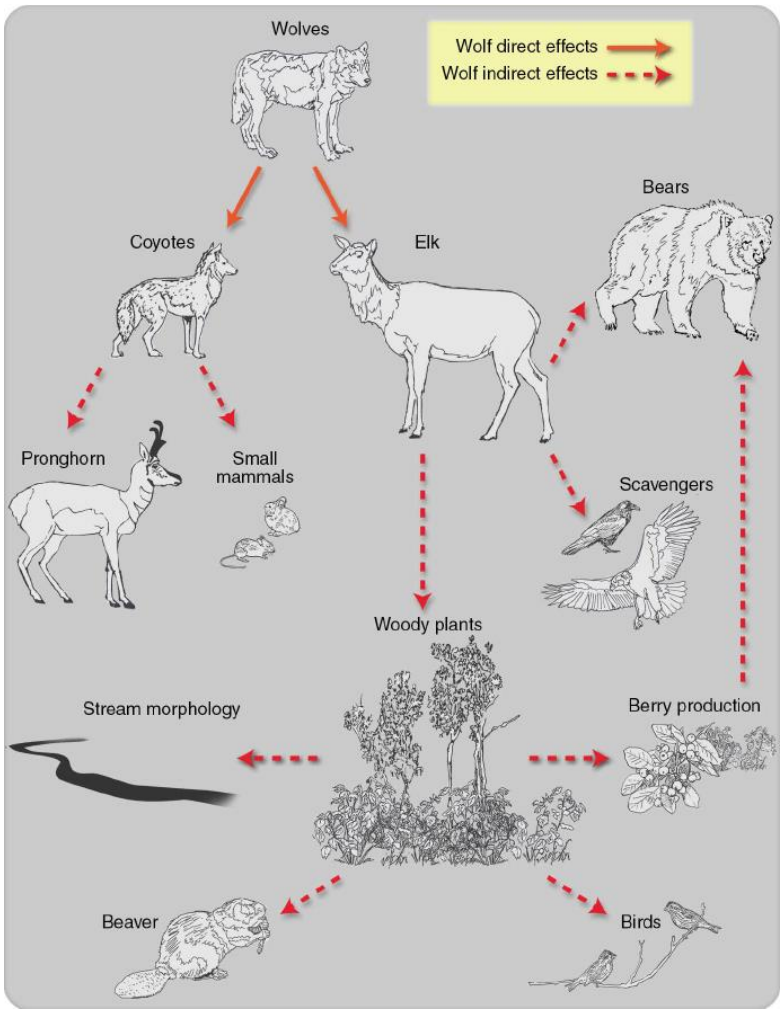

Figure 2: Conceptual diagram showing direct (solid lines) and indirect (dashed lines) effects of grey wolf reintroduction into the Greater Yellowstone ecosystem (source: Ripple et al. 2014)

3.1.3. Other impacts of large carnivores

Species of large carnivores do not only provide ecological services within their environment. Stable populations can provide source of income associated with tourism such as photo safari in Africa or wolf-related tourism in national parks in the United states. Activities associated with wolf-related tourism provide millions of dollars per year in income (Richardson & Loomis 2009; Ripple et al. 2014). Species like brown bear or grey wolf are among the most iconic and admired species on our planet. Their ability to attract public attention allows conservationists to use such popular species as 'umbrellas'. Therefore, they are often used as a cause to establish protected areas which serve to protect biodiversity within their range (Linnel, Swenson, Andersen, 2000).

One of the reasons why is managing populations of large carnivores very important is their high vulnerability to human activities. Their low population densities, low reproductivity, high nutrient demand, and their extensive range behaviour makes them very vulnerable towards to conflict with humans (Ripple et al. 2014). Competition between large carnivores and humans has been happening through history. Killing of livestock and occasional injuring or killing humans led to persecution of large carnivores in Europe and other parts of the world in the past two centuries (Morrison et al. 2007; Rigg et al. 2011). Even today conservation of these species can be met with negative public opinion and is it still challenging because of the socioeconomic factors (Skogen, Mauz, Krange 2006; Kaczensky et al. 2009).

Reintroduction of large carnivore species back to their historically inhabited areas has brought back conflicts with humans. Especially in rural areas, reintroduction of large carnivores can be met with strong negative views from local people. Such attitude towards large carnivores can be seen in many countries across Europe where adaptations for coexistence with apex predators and ability to protect livestock have been lost (Skogen, Mauz, Krange 2006). It is not only conflict between farmers and large carnivores which has risen. Conflicts between certain social groups have also emerged. People living in rural areas where carnivores have returned tend to have stronger negative opinion towards large carnivores than people living in areas where large carnivores are not present. This makes managing populations of large carnivore species not only an ecological issue, but also socioeconomic and even political (Breitenmoser 1998; Kaczensky et al. 2009; Arbieu et al. 2019).

3.2. Monitoring of large carnivores in Europe

Monitoring is defined as a process where results are repeatedly compared with previously defined goal. Term monitoring can be sometimes mistaken with survey, which is defined as single time collection of information through standardized procedures. Whereas monitoring is series of surveys to reach a certain goal. It can be divided into passive and active. Active monitoring consists of collecting data for certain purpose and aims to minimalize bias. Whereas passive monitoring uses data from public encounters and does not require field work. Public encounters may include traffic kills, direct observations, harvest data or damage reports. Use of passive monitoring can be strongly biased and might not be suitable for all monitoring tasks. Chosen monitoring method depends on many factors such as distribution of animals, their species or environment they inhabit. Some methods are more suitable for certain species more than other and there is not a method that would be applicable for all large carnivore species. Thus, use of combinations of methods is usually required to get more accurate results (Kaczensky et al. 2009).

3.2.1. Specifics to large carnivore monitoring

Management and conservation of large carnivores requires to know population size to evaluate population status to decide harvesting quotas or to obtain parameters for conservation principles such as the IUCN criteria for Red-listing evaluations (Kindberg et al. 2011). However, these species are typical for their low reproduction rates, long live spans, elusive behaviour and low population densities across large geographic areas, often in forested mountain regions (Mumma et al. 2015). After years of persecution, large carnivores have developed a propensity to avoid any contact with humans, therefore they are difficult to observe (Caniglia et al. 2012). Even though obtaining a reliable estimation of population sizes is very important for their management actions (hunting quotas), these species are particularly difficult and can be exceedingly expensive to monitor through traditional field methods (Kindberg et al. 2011; Caniglia et al. 2012). Nonetheless, knowledge of population sizes is often demanded by public and managers often have to face challenges of management measures (evaluating response to hunting, estimating population trends and sizes etc.) within short time spans (Kindberg et al. 2011).

3.2.2. Traditional methods of monitoring

Traditional monitoring of large carnivores includes methods such as direct observations, telemetry, camera trapping, present sign surveys (e. g. snow tracking, faeces or hairs identification,), aerial surveys, capturing live animals or use of scent stations (Arnemo et al. 2006; Kaczensky et al. 2009). These methods often depend on capturing and handling animals which disturb studied species in their habitat and can be dangerous for the animals (Solberg et al. 2006; Mumma et al. 2015; Woodruff, Lukacs, Waits, 2020). Although animal mortality due to capturing is relatively low. It is still an ethical concern especially when other less dangerous methods are available. Even low mortality rates can be crucial for very small populations of threatened species. (Arnemo et al. 2006). Capturing large carnivores can also be dangerous for the researchers thus, proper training and education of people handling the animals is required (Solberg et al. 2006; Woodruff, Lukacs, Waits 2020).

Present sign surveys and camera trapping have been probably the most used traditional monitoring methods of large carnivores in Central Europe. Even though these methods might not be as accurate as aerial surveys, but they are much less expensive (Kaczensky et al. 2009). However, studies in bear populations shows that, these methods appear to be less effective at large spatial scales, which is especially important in carnivore species because of their behaviour and distribution in remote areas. Survey done in brown bear population has also shown that traditional field methods tend to underestimate true size of the population, which makes them less reliable compared to properly applied genetic methods (Solberg et al. 2006; Barea-Azcón et al. 2007).

3.2.3. Non-invasive genetic monitoring

Development of molecular genetics, but also new statistical methods have enabled researchers with a great option to study animal populations (Schwartz, Luikard, Waples 2007, Skrbinšek et al. 2019). Genetic monitoring has proven to be one of the best choices to study populations over time and to evaluate if the studied populations need management action (Schwartz, Luikard, Waples 2007).

Genetic monitoring is usually understood as use of data collected using molecular markers to evaluate temporal changes in populations genetic metrics or other population data (Schwartz, Luikard, Waples 2007), however this term does not have precise

definition and its interpretation depends on field of study. Genetic monitoring can be divided into two categories. Category I. focuses on traditional form of population monitoring and consists of two subcategories. Category Ia focuses on identifying individuals and estimating their vital rates and abundance. Whereas category Ib focuses on identifying species and other groups (e. g. subspecies, genetically differentiated populations) and gives insight into changes in occupancy, presence of hybrids and occurrence of diseases and pathogens in populations.

Category II. provides information about evolutionary and demographic processes in studied populations such as changes in abundance, their effective population size, gene flow and population structure over time (Schwartz, Luikard, Waples 2007; Stetz et al. 2011; Caroll, et al. 2018).

3.2.3.1. Advantages of genetic monitoring

In comparison to traditional methods, genetic monitoring appears to have several benefits over traditional methods and appears to be more cost-effective in long-term monitoring of large carnivores. First benefit might be that traditional methods often depends presence of snow (Galaverni et al. 2012) or on visual detection, thus they are less effective in areas with dense tree cover where large carnivores are usually found (Stenglein et al. 2010). For instance, snow-tracking of wolves cannot distinguish wolves from dogs or hybrids whereas with genetic monitoring it is possible (Galaverni et al. 2012). Collecting non-invasive samples is also much less labour intensive than catching animals (Stenglein et al. 2010) and it does not need to be performed strictly by professional personnel (Kojola, Heikkinen, Holmala 2018). Results are also not biased due positive or negative animal response to trapping (Miller, Joyce, Waits 2005).

Probably the best feature of genetic monitoring is its ability to quantify temporal changes in population genetic metrics or other population data in elusive species and small endangered populations (Carrol et al. 2018). Permanent genetic markers can be used to track animals during their entire life (Waits & Leberg 2000). Therefore, data collected through genetic monitoring at different points in time allow scientists to better understand population dynamics and detect responses to management actions. That is particularly important in translocations and reintroductions to assess its success and to ensure probability of persistence (De Barba et al. 2010). We can also study recruitment of captive

individuals into wild populations and determine whether they are making reproductive contributions or not (Schwartz, Luikard, Waples 2007).

Many biological and demographic parameters (hybridization, occupancy, disease status etc.) have also been estimated through genetic monitoring and it is increasingly used to estimate responses to selective pressures such as climate change or exploitation (Carrol et al. 2018). It can also be used as an efficient tool to monitor genetic diversity in captive populations with small number of founders to prevent loss of genetic variation due to faulty breeding protocols, which would compromise recovery of species (Schwartz, Luikard, Waples 2007).

3.2.3.2. Disadvantages of genetic monitoring

Although use of non-invasive sampling appears to be very promising method for monitoring in large carnivore species. It is still a relatively new method and has few weaknesses (Waits & Paetkau 2005). First and probably one of the most important caveats to genetic monitoring are its high initial costs (laboratory equipment, genetic markers and optimalization of sampling methods) and added expenses associated with re-analysis of non-invasive samples (Schwartz, Luikard, Waples 2007; Qu & Stewart 2017).

Second limitation are genotyping errors which can be generated at every step of the process (sampling, DNA extraction, molecular analysis, scoring, data analysis; Bonin et al. 2004). Genotyping errors are particularly problematic for genetic census studies in comparison to other uses of genetic data because they can lead to either overestimation or underestimation of population size (Creel et al. 2003). Underestimation occurs when more individuals are identified as one, such error is called 'shadow effect' (Petit & Valiere 2006; Lampa et al. 2015). On the other hand, overestimation occurs when incorrect genotypes are identified as a new individual. False homozygotes are produced if only one allele of heterogenous individual is detected and amplified which it is called 'allelic dropout'. Whereas amplification of dinucleotide microsatellites can cause production of amplification artefacts which can be misinterpreted as true alleles. Such false allele might be detected if it occurs at a heterozygous locus (three alleles are present), however if false allele occurs in homozygous locus, then the individual is recorded as a heterozygote. Thus, re-analysis or comparison to blood or tissue samples needs to be done to discover genotyping errors and to distinguish correct and incorrect genotypes (Taberlet, Waits, Luikard 1999; Taberlet 1999).

Third disadvantage to genetic monitoring is risk of contamination. Non-invasive samples tend to be more vulnerable to contamination due to their low quantities of DNA. Thus, strict precautions need to be complied to reduce possibility of contamination, both in field and in laboratory. In field, samples should not be collected with bare hands (Waits & Paetkau 2005; Petit & Valiere 2006). In laboratory, extraction of DNA and polymerase chain reaction (PCR) should be separated. Proper cleanliness of laboratory and lab technique implemented by an educated lab technician also help to minimalize risk of contamination. (Waits & Paetkau 2005).

Fourth caveat to genetic monitoring is that non-invasive samples are more vulnerable to fraud because it is easier to replace samples than a live animal (Schwartz, Luikard, Waples 2007). Such fraud has already occurred, for example during survey evaluating distribution of Canada lynx (*Lynx canadensis*) in The United States (Mills 2002).

3.2.3.3. Non-invasive samples and their collection

Non-invasive samples such as hair or faeces provide source of DNA, which can be used to identify individuals and provides genetic data without disturbing animals (Schwartz, Luikard, Waples, 2007; Carrol et al. 2018). Use of non-invasive sampling was introduced as an alternative method to destructive sampling methods used in the past. These samplings often led to death of studied animals which is not desirable. First alternative to destructive methods was use of blood samples or feathers to study proteins. Revolution came with development of Polymerase chain reaction (PCR) which allows amplification of degraded DNA even at low quantity. After development of PCR, use of destructive samples was no longer necessary (Taberlet 1999).

Non-invasive samples are such samples, which an animal left behind and do not require catching or disturbing studied animal. Non-destructive sampling is sometimes improperly considered as non-invasive. Difference is that animals in non-destructive sampling often need to be caught in order to get samples (e.g. to pluck hair or feathers) and then released. Whereas in non-invasive sampling scientists often do not even see studied animals and use only samples which were left behind (Taberlet, Waits, Luikard 1999).

Non-invasive sampling in monitoring of wild animals was first used in 1992 (Carrol et al. 2018). It was introduced as an alternative method to collect genetic samples from brown bears in Pyrenees (Taberlet & Bouvet 1992), but also to study social structure in western chimpanzees (*Pan troglodytes verus*) in Africa (Carrol et al. 2018). Since then, non-invasive method has become very attractive for scientists and many studies have used only non-invasive samples as source of DNA (Taberlet, Waits, Luikard 1999). Currently, hair and scats are two most used sample types for genetic monitoring in large carnivores. However other samples such as saliva or urine are also available as source of DNA although they are often considered suboptimal (De Barba et al. 2012; Boitani & Powell 2012).

The reason why non-invasive samples have become so popular in monitoring of large carnivores is the possibility to obtain genetic material without need of tracking, catching or even seeing studied animals (Taberlet 1999). It has been applied in monitoring of numerous species and it is increasingly used to estimate abundance in species with large home ranges, which cannot be directly counted (Schwartz, Luikard, Waples 2007). Therefore, non-invasive sampling appears as a very promising solution to reliable monitoring of large carnivore species (Kindberg 2011; Carrol et al. 2018).

Sampling methods and study design are crucial factors, which can affect accuracy of the entire study (De Barba et al. 2010). Factors such as habitat-use, social structure or availability of sample material cannot be neglected in designing sampling strategy. Differences in quality and the quantity of DNA extracted from non-invasive samples among species also should be taken in consideration, e.g. wolf faeces provide much more DNA than bear faeces. Therefore, generalization between species is hazardous and can cause biased results (Taberlet, Waits, Luikard 1999).

Sampling can be conducted randomly, opportunistically or using standardized sampling design. Strategies for population size estimations are usually designed to maximise number of recaptures with high intensity sampling in a limited geographic area. However, behaviour, large habitats and high mobility of large carnivores make high intensity sampling quite difficult. Thus, collection of samples very often relies on work of local volunteers (hunters, tourists etc.) in order to collect as many fresh samples as possible within the large areas which would be otherwise very expensive (Kindberg et al. 2011; Skrbinšek et al. 2019). However certain number of professional personnel is still

required to achieve reliable estimation of population size in these species (Kojola, Heikkinen, Holmala 2018).

Collection and storage procedures are also very important (Carroll et al. 2018). Samples can be collected all year around, however certain time periods are more suitable for sampling sessions due to lower DNA degradation rates (Taberlet 1999; Agetsuma-Yanagihara, Inoue, Agetsuma 2017). After collection, samples should be dried in silica gel, frozen or stored in 95% ethanol or DET buffer to preserve DNA from degradation. Samples preserved in ethanol, silica or DET buffer can be stored in room temperature, however it is advised to freeze samples at -20 °C or colder to increase DNA yield (Kelly et al. 2012).

3.2.3.3.1 Faeces

Faeces are primary source of non-invasive DNA for monitoring of mammals (Lobo et al. 2015). Scat analysis is used in monitoring of many carnivores due to its quick application, large number of samples available and relatively cheap costs (Marucco, Pletscher, Boitani 2008). Large carnivores often deposit scats at prominent sites for intraspecific communication. These scats are then opportunistically collected by volunteers or by walking transects. Efficiency of sampling can be increased by tracking animals (via tracks in snow, mud etc.) and using dogs trained to detect scats (Kelly et al. 2012), which allow quick and efficient way to collect samples and do not require any attractants (Long et al. 2007).

Faecal DNA extracted from scat samples originates from cells sloughed from the intestinal lining and is usually degraded and scarce. In addition, faeces also contain DNA of prey animals and PCR inhibitors (Hausknecht et al. 2007; Lobo et al. 2015). DNA quality also varies depending on environmental conditions and location of collection (Marucco, Pletscher, Boitani, 2008; Kelly et al. 2012). Environmental conditions (rainfall, temperature, humidity, UV etc.) increase degradation rates and so does time between defecation and collection of the sample. Temperature and precipitation play the most important role in collecting faecal samples. Significant DNA degradation occurs approximately after 6 days in winter, however in summer, such degradation occurs after 3 days. Thus, it is important to collect samples as fresh as possible to minimize the DNA degradation (Agetsuma-Yanagihara, Inoue, Agetsuma 2017). Not collecting samples

soon after deposition leads to increase of genotyping error rates and decrease of amplification success (Lobo et al. 2015).

3.2.3.3.2 Hair

Hair sampling is used especially in monitoring of ursids and felids and even canids. Although only few surveys have included canids as their primary target for hair sampling. Currently, it is the most effective method in monitoring of wolverines in Scandinavia (Kendall & McKelvey 2008).

Hair sampling is often combined with camera-trapping (Figure 3) to provide complementary information about morphology of the sampled individuals or wolf pack structures (Canu et al. 2017). Efficiency of this method differs depending on local conditions and studied species (Schmidt & Kowalczyk 2006).

Figure 3: Lynx rubbing on a barbed pad (photo by P. Nyland) and a bear passing over barbed wire (photo by S. Himmer, Arctos Wildlife Services and Photography) adopted from Kendall & McKelvey (2008)

Samples are obtained through hair snags or rub pads which are both quite inexpensive. First method uses bait (blood, rotten meat etc.) which attracts animals and barbed wire is placed around the bait. Animals then leave their hair on barbed wire when they are approaching or leaving the bait. Other approach uses rubbing behaviour of

animals. Rubbing behaviour is considered as a form of communication in many species, especially in large solitary carnivorous species such as bears and lynx. This behaviour may be related to territory marking or sexual activity. Therefore, hair collection using rub pads during mating seasons might increase number of samples collected (Schmidt & Kowalczyk 2006; González-Bernardo et al. 2021). Rub pads are placed on trees or other structures which are able to detect animals not sampled by barbed wire. However, specific species attractants are usually required to lure animals to the device and to make them to rub against its surface (Kelly et al. 2012).

Hair samples can also be collected via transect samplings, where volunteers search for hairs on trails and their surroundings within animal range. However, transect sampling is more suitable for collecting faeces than hair samples (De Barba et al. 2012).

During analysis it is possible to pool multiple hairs to increase DNA yield for species detections. Multiple hairs with follicles usually provide higher quality DNA than faeces and have less agents that inhibit and prevent DNA amplification. However pooling multiple hairs is risky for identifying individuals because it can create false new genotypes. Thus, only one hair with follicles, which usually yields less DNA than faeces, should be used for analysis to prevent creating of a false genotype. Although it might be possible to use multiple hair samples using a hair snag device that allows only one animal to be sampled (Kelly et al. 2012).

3.2.3.3.3 Urine

Urine samples can be collected with swabs, but they are usually collected directly from snow as a snow-urine mixture and then frozen until DNA extraction (Kelly et al. 2012). Application of urine sampling in monitoring of wild populations has a few benefits. First benefit is that urine samples contain predominantly DNA of the correspondent individual and samples are not affected by DNA of prey animals as observed in scats. In addition, urination frequency is much higher than defecation frequency. Nonetheless, use of urine as primary source of DNA in monitoring is usually limited by climate and geographic conditions. However, other sample types are also limited by fast decompositions during warm temperatures (Hausknecht et al. 2007). Urine samples also show slightly lower amplification success than scat samples and results might be biased due to urine marking behaviour of animals (Hedmark et al. 2004; Hausknecht et al. 2007).

3.2.3.3.4 Saliva

Saliva serves as an excellent source of DNA because it contains many cells and has high amplification rates. Study from Lobo et al. (2015) shows that using of saliva as source of DNA could allow detection of socially low-ranked individuals not involved in marking behaviour (urine, faeces) at prominent sites. However, it is considered as suboptimal source of DNA in monitoring of wild populations due to insufficient methods to systematically obtain samples. Saliva could serve as a feasible sampling tool to supplement hair and scat sampling particularly in ecosystems where bears feed on spawning salmon (Wheat et al. 2016). However, collection of saliva in other areas would require tracking animals and obtaining saliva from recently killed prey or other food remains. An alternative might be a bait made from porous material that would attract an animal and absorb saliva after licking or chewing, however such bait has been used only in laboratory conditions (Lobo et al. 2015).

3.3. Capture-Mark-Recapture method

Capture-mark-recapture (CMR) method was originally applied on small carnivores (coyotes) but appears so be very useful in monitoring of many carnivore species (Gese 2001). It has become is one of the most common methods used for population monitoring (Woodruff, Lukacs, Waits 2020). There are usually multiple sampling sessions applied to estimate population size.

Principle of this method is capturing live individual, marking this individual (radio collars, ear tags, dyes, microchips physiological markers etc.; Gese 2001) and releasing the animal in the first sampling session and recapturing in following sessions. Population size N is estimated in following sessions from ratio of captured individuals, who were already marked, to number of unmarked individuals. Elusive behaviour of some species, thus low catchability led scientists to use of molecular tags from non-invasive samples (faeces, hairs) to study animals that are neither seen nor captured. Extracted DNA from these samples is amplified at certain number of microsatellite loci which serve as a tag for each individual (Petit & Valiere 2006; Lampa et al. 2015). Microsatellites are used as markers in most studies, however other markers such as single nucleotide polymorphism (SNP) can be used to identify an individual (Lukacs & Burnham 2005). Genetic profiles can serve just like the traditional tags (e. g. radio-collars) and they permanent, thus they

can be used through the entire life of the animal (Taberlet 1999). New genetic fingerprint serves as a ‘mark’. ‘Recapture’ is recorded when the same genotype is recorded from a different DNA sample (Pearse et al. 2001). Molecular tags also allow scientists to estimate population size N in just one session from the asymptote of accumulation curves where plots are the number of unique tags against total number of samples (Petit & Valiere 2006; Lampa et al. 2015).

CMR method appears to be cost effective for relatively small populations thus it is suitable for estimating population sizes of large carnivores, which usually occur in populations up to few thousands of individuals. Estimating abundance in populations over this size would require sampling and analysing large number of samples which would be very expensive and time consuming (Lukacs & Burnham 2005).

Choice of capture-mark recapture model is very important and it decides what can the result be used for. There are two main models for CMR and one model which combines the first two (Lettink & Armstrong 2003). Both of these models have their roots up to sixteenth century, but they have become more complex during second half of the twentieth century (Pollock 2000; Lindberg 2012). First model is applied in so called closed populations, where we assume that the size of the population does not change during the study (no births, deaths nor migration). Second model is for open populations, where births, deaths, immigration and emigration occur between sessions and are taken in consideration (Lettink & Armstrong 2003). Combination of open and closed design is called Robust design (Lindberg 2012).

3.3.1. Closed population model

This model is used to estimate abundance of species, i.e. number of individuals in specific time and space. Closed population model is sometimes called Peterson-Lincoln estimate after two scientists who independently developed this method to estimate size of animal populations. It needs to be applied in at least two sessions, but more sessions are usually applied, since more sessions provide more data, thus, more reliable estimates (Lettink & Armstrong 2003; Lindberg 2012). If more than two sessions are applied, then new individuals in subsequent sessions are also marked if it is not the last session of the study (Lindberg 2012).

$$\widehat{N} = \frac{n_1 \times n_2}{m_2}$$

$\widehat{N}$= estimated population size

n_1= number of animals caught in the first session

n_2= number of animals caught in the second (subsequent) session

m_2= number of marked animals recaptured in subsequent session

Figure 4: Population size estimation in two sessions (source: Lettink & Armstrong 2003)

Certain assumptions need to be complied for this method to create a reliable estimation of population size. First is the assumption of closure which means that there are no additions or deletions during the study. Although this assumption is impossible to achieve in wild populations because all populations are subject to these processes, it is possible to assume population closure if the sessions are applied during certain limited time periods and if the survey covers area large enough so there is smaller chance of individual leaving the area during sampling session (Lettink & Armstrong 2003; Kindberg et al. 2011). Second assumption requires all animals in the study to have the same chance of being caught. Third assumption requires markers to prevail during the entire study (Pollock 2000; Lettink & Armstrong 2003).

There have been two extensions of closed population model to address issues with data based on DNA samples. First extension includes a parameter to estimate genotyping error. Error is estimated using the unequal number of genotypes only observed once relative to genotypes seen more than once. Second method uses data available when

multiple samples from one individual are collected within an occasion. These data are used to account for individual heterogeneity in capture probability (Lukacs & Burnham 2005).

3.3.1.1. Application in bear populations

Closed population model appears to be suitable for estimating sizes of bear populations. Although bears move across large areas, assumption of closure can be fulfilled if sampling session is no longer than 12 weeks and if it takes place within hyperphagia period (starts in late summer and ends in fall; Fitz 2017), when there is little immigration or emigration (Kindberg et al. 2011; Skrbinšek et al. 2019). This model was applied by Kindberg et al. (2011) to estimate bear abundance in Sweden between years 2001 and 2008, it showed population growth and approximate population size of 3,298 (2,968–3,667) individuals in 2008. This estimation corresponds with official numbers from Swedish Institute, which assumes around 3,200 individuals to be found in Sweden (Lagerberg 2019). Ciuci et al. (2015) used similar approach to estimate size of small bear population in Central Italy. Although hair sampling occasions took place from June to September (more than 12 weeks), population closure was assumed based on relatively isolated nature of this population and previous data from radiotelemetry. Sampling took place between years 2008 and 2011 and they estimated population size of 51 (47–66) individuals in 2011, which showed that the number of individuals in this population was not declining and slightly growing.

Minimum yearly bear population size (after mortality, before reproduction) in Slovenia was also estimated using closed population model by Skrbinšek et al. (2019) in 2007. In this case sampling took place from September till November (to avoid hunting season from June till early September) and the estimation had to be corrected for the edge effect caused by transboundary nature of this population where individuals move across Slovenian-Croatian border. Size of 2007 "winter population" was estimated to be 424 (383–458), which was lower than previous official estimates that assumed this population to consist of 500–700 individuals.

3.3.2. Open population model

Studies often takes many years to document population trends, which is especially important in newly colonized areas (Marucco et al. 2009). Thus, closed population

models are not applicable in such cases, because they assume no additions or deletions in populations during the study. This led to development of open population model (Pollock 2000), which is used if numbers of individuals change during the study.

$$\widehat{N}_i = \frac{n_i}{\hat{p}_i}$$

$\widehat{N}i$= population size for capture occasion i

n_i= number of animals captured on occasion i

$\hat{p}_i$= capture probability on occasion i

Figure 5: Population size estimation on occasion i (source: Lettink & Armstrong 2003)

Open population model data is much more complicated than in closed population model because of the processes, such as births, deaths or migration that are changing number of individuals and need to be taken in consideration (Miller, Joyce, Waits 2005). In comparison to closed population model, open population model generally provide more robust density estimates with fewer biases (Whittington & Sawaya 2015).

Open population model requires several assumptions to be applied to provide reliable results in population studies. First original model was independently introduced by Jolly (1965) and Seber (1965; JS model), it required the same capture and survival probability for all animals in study area. However, it is known that in real populations, individuals do not have the same capture probability (Miller, Joyce, Waits 2005). Thus, this model would be often biased, because certain groups have higher survival chance than other groups, e.g. adult individuals have higher survival rates than juveniles. The original JS model was improved to Cormack-Jolly-Seber (CJS) model (Cormack 1964; Jolly 1965; Seber 1965), which has allowed scientists to divide animals into smaller related groups (animals with the same sex, age, weight etc.) to reduce bias. Therefore, one of the assumptions for open population model is that all animals in the same group have the same chance of survival between sampling occasions and the same probability of capture each sampling. Another assumption is that marks are not lost during the length of the study (Pollock 2000; Lettink & Armstrong 2003; Lindberg 2012). These

assumptions make it possible for open population model to estimate birth rate, survival rates and size of population for almost all samples (Pollock 2000).

Estimating capture probability and survival probability is crucial for this method. Each captured animal has probability of surviving to the next recapture session and each alive animal has probability of capture on next occasion. However dead and undetected animals are indistinguishable at the time of the session and further sessions are required to estimate capture probability and probability of survival (Lettink & Armstrong 2003). Once capture probability is known, population size can be estimated, whereas survival probability is important to estimate recruitment between sessions (Pollock 2000; Lettink & Armstrong 2003; Lindberg 2012).

3.3.2.1. Applications in Europe

Open population model is well suited for long-term monitoring of mobile and elusive species where we cannot assume population closure (Marucco et al. 2009). Therefore, this model has been applied in studying populations of wolves, bears, wolverines, and even Eurasian lynx, however in monitoring of lynx the main source of data for population estimation is usually obtained through camera–trapping and genetic monitoring has been applied only occasionally (Interreg Central Europe 2018). Grey wolf is an example of species where we cannot assume population closure. Its populations are intrinsically open due to wide home ranges which are potentially connected by long-range disperses, therefore we usually cannot apply closed population model (Caniglia et al. 2012).

Caniglia et al. (2012) applied open population model to study trends in wolf population in northern Italy. Samples were collected between years 2002 and 2009 and results showed population size growth. Mean annual size ranged from 117 individuals in 2003 to 233 individuals in 2007 with a mean annual finite rate of increase $\lambda=1.05\pm0.11$. Marucco et al. (2009) estimated very similar annual finite rate of increase in their study on wolf population size in the western Alps between years 1999 and 2006. Over the years, population grew from approximately 21 individuals in 1999 to 47 individuals in 2005, however in 2006 the population size decreased, probably due to poaching. Bishof et al. (2020) used open population model in monitoring of wolves, bears and wolverines in Scandinavia. Monitoring of Scandinavian population is particularly problematic due to

different conservation approaches in Sweden and Norway, therefore most of the animals are found in Sweden. After 7 years of monitoring of these species, they estimated population sizes in Scandinavia to be 2,757 bears, 375 wolves and 1,035 wolverines in 2018. Open population model was also applied to estimate size of bear population in Greece which is part of Dinara–Pindos bear population that is expanding towards the south. Mean number of individuals in Pindos mountains after 3 years of monitoring was estimated to be 182 individuals (Karamanlidis et al. 2015).

3.3.3. Robust design

So called robust design combines both open and closed population models. One of the advantages of this design is, that it allows animals to join into or leave study population (Lukacs & Burnham 2005). With this design, scientists are able to separate recruitment from immigration, estimate temporary emigration or allow for unequal catchability (Pollock 2000). This design uses sampling occasions from open population model as primary occasions, which are subdivided into shorter closed population model occasions, which are referred as secondary occasions (Figure 6; Miller, Joyce, Waits 2005).

Time periods between primary occasions are usually long and population is assumed to be geographically and demographically open (birth, death and migration occur). Secondary sampling occasions have very short time intervals and are used for better estimating capture probability. Populations are assumed to be closed between secondary sampling occasions. In robust design capture probability is estimated in secondary occasions (closed population model), which allows estimating of abundance for each primary occasion (open population model). This design also allows estimating survival rates, temporary emigration and immigration between primary periods (Lukacs & Burnham 2005; Miller, Joyce, Waits 2005).

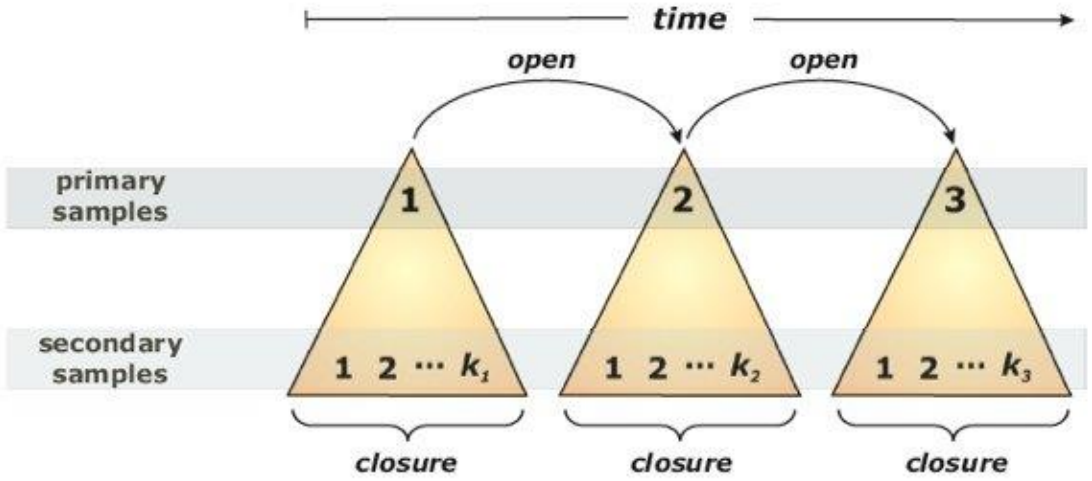

Figure 6: Basic structure of the Pollock's robust design (Source: Bouveroux & Mallefet 2010)

There has been an extension to reduce bias caused by genotyping errors. It estimates genotyping error rates at each primary sampling occasion. Thus, results of abundance and survival rates are more accurate. If genotyping errors would not have been taken into account, survival rates would be biased low and on the other hand, abundance would be biased high (Lukacs & Burnham 2005).

Robust design was applied by Marucco et al. (2012) in Italian Alps to study wolves (to estimate population size, number of wolf pack, distribution and effective population size). They applied open capture-recapture sampling design where each transect was covered multiple times during each sampling session to estimate mortality rate and population size. At the same time, they defined multi season occupancy design, which fitted into the open CR sampling design. Each cell (5 x 5 km) of the study area was surveyed at least once per month and it was repeated four times each winter to estimate occupancy and distribution. Average number of surveys per season was 28. The minimum population size increased over 11 years of the study from 20 to 61 in early winter and from 17 to 52 in late winter with 1.20±0.28 annual rate of increase. Number of packs in this region have increased from 1 to 18 since 1994. Marucco et al. (2012) also recommends using winter population size for management purposes because it is more conservative.

3.3.4. Capture-Mark-Recapture software

Population estimations have benefited from improvement of field methods, but also from development of analysis methods using CMR software (Kindberg et al. 2011). Many software programs have been developed to compare models and to help scientists in estimating population sizes. Such software designed to help analysing capture-mark-recapture data can be for example programs MARK, CAPTURE *capwire*, M-SURGE or POPAN (Lukacs & Burnham 2005; Miller, Joyce, Waits 2005). Program MARK is probably the most used software among these today and can be used both in open and closed population models. Difference between MARK and an older program CAPTURE is, that MARK considers differences in capture probabilities caused by biological characteristics of animals (same sex, age etc.), whereas CAPTURE uses only random variation. MARK also includes models designed to estimate genotyping error rates. Therefore, MARK has taken place of the older program in population estimations. Both programs M-SURGE and POPAN were designed only for open population models (Lettink & Armstrong 2003; Lukacs & Burnham 2005).

To address specific needs of using DNA samples in capture-mark-recapture, program *capwire* was developed. Difference between traditional capture-mark-recapture and DNA sampling is, that in DNA sampling, individuals might leave samples in several locations during one sampling session. This means that one individual can be captured multiple times during one session, thus analysing method must have been modified. *Capwire* model was developed specifically for DNA sampling and has two methods to estimate population size. First method sees population as an urn where individuals are continuously mixing and contains individuals with two different capture probabilities (high and low). Such urns are usually social animal groups like canid packs, ungulate herds, or primate troops. More common than urn is the second model where individuals occupy semidiscrete areas. This method is based on DNA deposition patterns and heterogenous movements in occupied space. Such species are for example ursids with their big home ranges (Miller, Joyce, Waits 2005).

4. Aims of the practical part of the thesis

- To create an estimation of population size using dataset of wolf samples

5. Material

We used data set of wolf samples from diploma thesis of Bc. Kamila Valentová which is focused on conservation of wolves in the Czech Republic. Data set contained 184 samples which were collected by several organizations such as Friends of the Earth Czech Republic, Nature Conservation Agency Czech Republic, Ministry of the Environment of the Czech Republic, Czech Environmental Inspectorate and research project OWAD. Sampling took place in the Czech Republic between years 2014 and 2021.

Majority of the collected samples consisted of faecal samples however other sample types such as hair, saliva, tissue, blood or urine were also analysed. Samples from this dataset were processed by group of scientists from Czech University of Life Sciences, University of Ostrava and Charles University who research genetics of large carnivores. I did not personally analyse these samples, nonetheless I have isolated DNA from other samples.

6. Methodology

For DNA isolation from samples were used silica-binding extraction kits. QIamp Fast DNA Stool Mini kit (Qiagen) was used for extraction from faecal samples and for tissue samples was used DNeasy Blood & Tissue Kit (Qiagen). We precisely followed manufacturer's instructions of the isolation kits, except the last step. Instructions recommended using 200 µl ATE Buffer to wash away impurities from the silica membrane where DNA is bound. Instead, we repeatedly (2-3 times) used 50 µl of ATE Buffer to wash out the membrane in order to increase DNA yield. Purified DNA and ATE Buffer were then measured in spectrophotometer (Nanodrop, ThermoFisher Scientific) to determine the concentration of nucleic acids.

Before PCR could start, we had to add Multiplex PCR polymerase, fluorescently labelled primers and RNA-free water to the mixture. In this study, we used 18 wolf microsatellite loci to determine identity of individuals (ATHk211, CPH5, CXX279, FH2001, FH2010, FH2054, FH2087, FH2088, FH2096, FH2097, FH2137, FH2140, INU055, INRA21, REN169D01, REN169O18, REN64E19, VWF). Microsatellites consist of motifs of 1-6 nucleotides repeated several times and are considered as powerful genetic markers due to their mutation behaviour and high variability (Guichoux et al. 2011; Kraus et al. 2015). Each locus has 'reverse' and 'forward' variant of primer and one of them is always fluorescently marked which provides detection sensibility of amplified DNA. DNA was amplified at 18 microsatellite loci in PCR, which is a three-step (DNA denaturation, primer annealing, polymerase extension) thermal cycling process where number of DNA strands is doubled after each cycle (Schochetman, Ou, Jones 1988).

After obtaining PCR product, we added formamide and ladder 500 LIZ® Size Standard (ThermoFisher Scientific) to conserve single stranded DNA. Final products were sent to Faculty of Science at Charles University for fragmentation analysis. Personally, I did not analyse sufficient number of samples to create an estimation of wolf population size in the Czech Republic. Therefore, for our estimation, we had to use data set of wolf samples from diploma thesis of Bc. Kamila Valentová, which is focused on conservation genetics of grey wolves in Central Europe.

Individuals in provided data set were already identified. It contained 184 wolf samples with 156 unique genotypes. We counted numbers of genotype repetitions in each wolf season, which starts on 1st May and ends on 30st April. We also counted repetitions of genotypes for all seasons together (Table 1).

Table 1: Numbers of sampled individuals and their repetitions in the data set.

Season 0 (2014-2017)		**Season 1 (2017-2018)**	
Repetitions	N. of individuals	Repetitions	N. of individuals
1	12	1	11
2	1	2	1
3	1	3	0
4	0	4	2
Season 2 (2018-2019)		**Season 3 (2019-2020)**	
Repetitions	N. of individuals	Repetitions	N. of individuals
1	34	1	68
2	1	2	4
3	0	3	0
4	0	4	0

All seasons together	
Repetitions	N. of individuals
1	136
2	15
3	4
4	0
5	1

Population size was then estimated in software *capwire*, which is a package in R program developed specifically for estimating population sizes from non-invasive samples. We followed *cawire* tutorial from Pennel & Miller (2012). Data had to be entered as a two-column data frame. First column contained capture classes (repetitions) and the second column contained number of individuals in each capture class as shown in Table 1. We used 2 estimation models from Miller et al. (2005) available in *capwire* to estimate population size. In both models we had to specify maximum population to generate upper bound for the purposes of optimization (Pennel & Miller 2012). We chose

argument max.pop to be 1000 individuals, therefore it was much larger than reasonable expectation of population size which was assumed to be very small. First model used for estimation was Equal Capture Model (ECM). This model assumes that all individuals have the same capture probability, and that the population can be modelled as an urn where individuals are mixing. Second model used was Two-Innate rates model (TIRM) in which we assume that population contains a mixture of individuals with two distinct capture probabilities.

7. Results

Table 2: Estimated population sizes and model likelihoods.

Season 0 (2014-2017)	Estimated population size	Likelihood
ECM	13–48	-32
TIRM	13–70	-28
Season 1 (2017-2018)	**Estimated population size**	**Likelihood**
ECM	14–23	-45
TIRM	14–37	-41
Season 2 (2018-2019)	**Estimated population size**	**Likelihood**
ECM	35–618	-99
TIRM	35–637	-97
Season 3 (2019-2020)	**Estimated population size**	**Likelihood**
ECM	72–687	-269
TIRM	72–724	-263
All seasons together	**Estimated population size**	**Likelihood**
ECM	156–594	-842
TIRM	156–692	-808

In Table 2 we can see population sizes and their likelihoods of used models estimated in software *capwire*. We can see intervals where the lower numbers represent number of sampled individuals and the higher numbers represent maximum likelihood estimation for the population size. Population sizes were estimated for each wolf season and then for all seasons together (2014-2021). In Season 4 (2020-2021) we were not able to estimate population size because we did not have data from the entire season (1.5. 2020–30.4. 2021), last sample was collected 12.3. 2021. There were no recorded repetitions in available data at the time of our estimation, thus our data was not informative.

We can see that ECM shows slightly lower estimations and has lower likelihood whereas TIRM shows bigger population size estimations and more importantly higher likelihood, which is a statistical function measuring goodness of fit of statistical models. It is often used to generate estimators, where we usually want the estimator to have maximum likelihood (Glen 2021).

8. Discussion

Size of wolf population in the Czech Republic has been a sharply increasing since 2014 and there have been multiple recordings of reproducing wolves in the Czech Republic since then (OWAD 2021). Although the sharp increase in population size might also be influenced by increased sampling efforts in recent years, which can also be seen in Table 1 and Table 2. According to Ministry of the environment (ME) and AOPK (2021), there were 18 wolf territories in 2019 which were at least partially stretching to the area of the Czech Republic. Most of these territories are transboundary, however 13 wolf packs, which usually consist of 4–6 individuals in our conditions, and 3 couples were recorded in the Czech Republic (Figure 7) which accounts for about 70 to 80 individuals (Šůlová 2020; Lososová, Kouřilová, Soukupová 2021).

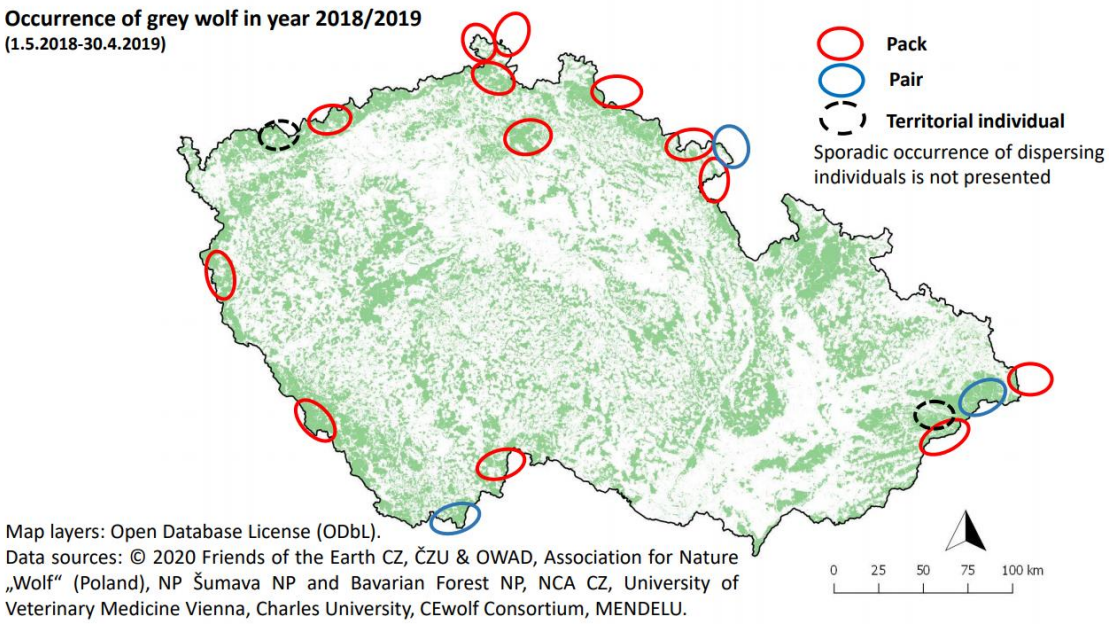

Figure 7: Occurrence of grey wolf in year 2018/2019 based on field monitoring (source: AOPK 2020)

Our estimation was only experimental because we did not have enough data. Nonetheless, we can see that TIRM has slightly higher likelihood, therefore this estimation model might be more suitable for future estimating of wolf population size in the Czech Republic.

Our results were much higher than expected, especially in Season 2, Season 3 and when we pooled All seasons together and significantly higher than estimation based on

field monitoring from 2019. Therefore, we can assume that our estimated population size was firmly overrated. Season 0 and Season 1 were not that extremely overestimated like other seasons however we can see quite a big drop in estimated population size between these two seasons which is to our knowledge unlikely and it was probably caused by very small number of samples analysed (Table 1).

There are several possible factors which might have caused overestimation of our results. Probably the most important factor affecting our results is that we had either very small number of samples available for our estimation or low repetitions of genotypes which are both crucial factors for accurate estimating population sizes through CR methods (Pearse et al. 2001).

Second factor affecting accuracy of our estimation is a fact, that most of the wolf territories in the Czech Republic are transboundary and animals are moving in and out of the Czech Republic freely (Šůlová 2020). However, our samples were collected only on the Czech side of the borders, which means that our samples were only portions and did not reflect the entire populations. Therefore, we would recommend future transboundary co-operation in order to obtain accurate estimations of population sizes.

Third factor which might have affected our results is that we perceived all animals in the Czech Republic as part of a single population. Nonetheless, Czech Republic as a region in Central Europe which is often considered as a crossroad and contact zone of several wolf populations (Figure 8; Hulva et al. 2018). According to haplotypes of individuals in our data set and study from Pilot et al. (2010), majority of our samples were obtained from individuals from Central European population which is expanding to the Czech Republic from north (Poland, Germany), however there were also samples belonging to individuals from Italian-Alpine population which is expanding from south (Austria, Germany), from Carpathian population which is expanding from east (Slovakia), and 2 individuals were identified as part of Baltic population. On that account our results might also be biased due to pooling multiple distinct populations into single population.

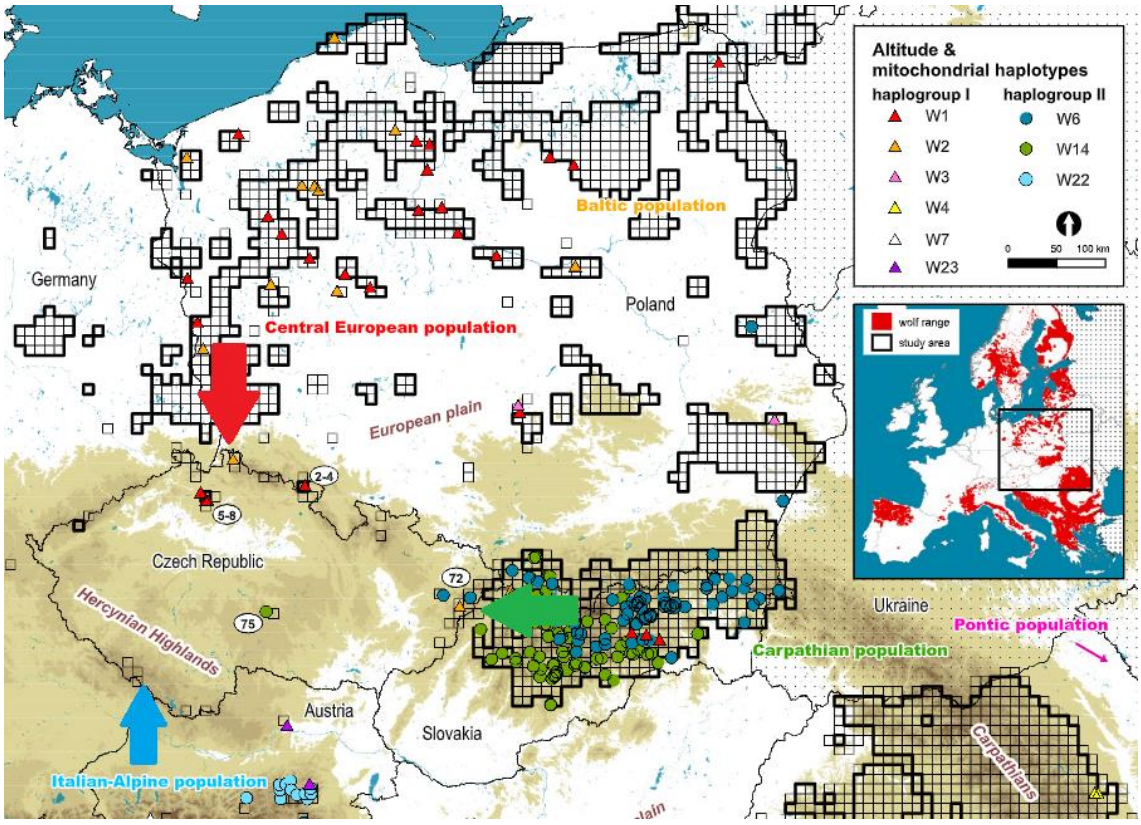

Figure 8: Populations of Grey wolf in Central Europe (source: Hulva et al. 2018, edited by author)

Therefore, unequal dispersion of wolves in the Czech Republic (Figure 7) should also be taken in consideration in future designing of monitoring programs and estimating population sizes could be done on regional level to increase accuracy of results, therefore we would avoid pooling multiple populations together. Although temporary occurrence of wolves in the majority of the Czech Republic, thus mixing populations in the future cannot be ruled out due to increasing numbers of wolves and their high mobility (OWAD 2021).

Last, but not least possible factor affecting our results might have been occurrence of genotyping errors, although analysis of samples was done by professional personnel and samples were usually analysed multiple times, thus we can assume that genotyping error rates are very low.

9. Conclusions

From the literature research we can see that estimation of populations sizes of large carnivores is very complicated, but also very important. We can assume that it will be even more important in the future if the trend of population growth in large carnivores will continue, which will also increase number of human-wildlife conflicts, therefore an increasing need for management actions.

Genetic monitoring has many benefits and appears to have great a potential for application in large carnivores and it has been already applied in several countries in Europe. However, although its results are supposed to be more robust and accurate, they can be strongly biased if the monitoring is not conducted accordingly. Therefore, strict measures should always be adhered at every step of the process in future applications to reduce chances of possible bias in results. There are several methods available to estimate population sizes, however not all methods are suitable for application in all species or all geographic regions. Thus, all possible factors should be taken in consideration and proper research should be always performed before application.

Although estimation of population size was only experimental, on that account it does not reflect real numbers of wolves in the Czech Republic and cannot be used as source of data to any form of management action. Our estimation provided us an insight into problematics of wolf monitoring in the Czech Republic and application of non-invasive sampling under capture–recapture modelling framework. Which could be very valuable in possible future designing of wolf census studies in the Czech Republic.

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