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Genetic Monitoring of Grey Wolf (*Canis lupus lupus*) in Central Europe

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled Genetic Monitoring of Grey Wolf (*Canis lupus lupus*) in Centra Europe 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 18.08.2023

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Vojtěch Zeman

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Abstract

The number of wolves (*Canis lupus*) in the Czech Republic has been increasing due to the ongoing expansion of populations from neighboring countries. The Czech Republic is considered a genetic crossroad as individuals from multiple populations come into contact in this area. In this thesis, non-invasive genetic monitoring was implemented to assess the information about the presence of wolves in the Czech Republic and its surrounding areas and to determine their population origin. A total number of 589 samples was analyzed between January 2020 and April 2023 and genotyped using 20 microsatellite loci with a genotyping success rate of approximately 50%. In total, 183 unique wolf genotypes were identified, and no wolf-dog hybrids were detected. Population affiliation was determined by the Bayesian Clustering Analysis using three comparative datasets of wolves with known origins (Alpine, Central European, and Carpathian populations). The majority of individuals (115) clustered predominantly with the Central European population, and the remaining 68 individuals were assigned predominantly to the Carpathian population. None of the 183 individuals appeared to have an exclusive origin in the Alpine population. Principal Coordinates analysis further supported the dissimilarity of the Alpine population from the tested genotypes. Individuals from the Central European population displayed lower genetic diversity compared to the individuals assigned to the Carpathian population as they exhibited lower values for the number of effective alleles, fixation index, and heterozygosity while maintaining a higher coefficient of inbreeding. The spatial distribution of samples revealed wolf presence in most of the mountain ranges along Czech borders and indicated that a certain level of gene flow is present between the populations. This was further supported by the discovery of long-distance movements between Javorníky Mts. in Slovakia and Orlické Mts. in the Czech Republic.

Keywords: *Canis lupus*, DNA analysis, genetic monitoring, microsatellites, non-invasive samples, population

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List of the abbreviations used in the thesis

CE – Central European

CMR – Capture-Mark-Recapture

DNA - Deoxyribonucleic acid

eDNA - Environmental DNA

HWE - Hardy-Weinberg equilibrium

iDNA - Invertebrate DNA

IUCN - International Union for Conservation of Nature

MCMC – Markov Chain Monte Carlo

mtDNA - Mitochondrial DNA

NGO – Non-governmental organization

NGS – Non-invasive genetic sampling

PCR – Polymerase chain reaction

Rcf - Relative centrifugal force

SNPs – Single-nucleotide polymorphisms

STRs – Short tandem repetitions

1. Introduction and Literature Review

1.1. Wildlife monitoring

Wildlife monitoring is an essential component of biodiversity conservation (Zwertz et al. 2021). It can be described in many ways, however, the most accepted definition is the use of repeated surveys to obtain data on wildlife population characteristics. These surveys encompass aspects such as spatial distribution, abundance, density, or other population parameters along with changes in status. Such data play an important role in evaluating management activities (Sauer & Knutson 2008; Kindberg et al. 2009; Marucco & Boitani 2012).

There are multiple compelling reasons to engage in ongoing wildlife monitoring, as the evaluation of data concerning wild populations can yield a wide range of benefits (Caughley 1977). Such reasons might include the need to assess information about invasive or pest species that might be harmful to biodiversity and natural resources as well as being a possible health hazard in the form of disease transfer to livestock and even to humans (Engeman & Witmer 2000; Ruffel et al. 2015). Monitoring game species populations is essential to prevent both over-harvesting and overabundance, thus ensuring the sustainability of viable populations (Morelle et al. 2012). Additionally, monitoring serves conservation purposes, preventing population declines and extinctions. For this purpose, it is necessary to obtain information about population parameters and trends to implement effective management actions (Witmer 2005; Marucco & Boitani 2012). Data generated from monitoring efforts can be utilized across various scales, ranging from the local, on-site level to the global levels (e.g., as indicators for global biodiversity goals, the IUCN Red List of Threatened Species, the CITES Appendix status of species; Zwertz et al. 2021).

As the world is witnessing the loss of species at an alarming rate (Butchart et al. 2010, Waldron et al. 2017) with hundreds or perhaps even thousands of species around the globe facing extinction every year (Chivian & Bernstein 2008; Ceballos & Ehrlich 2018; Hardulak et al. 2020), the role of conservation monitoring is increasing. Detection of trends in distribution and abundance in threatened species is crucial to determine

threatening processes as well as to evaluate the effectiveness of conservation efforts (Robinson et al. 2018). For this purpose, it is often necessary to monitor changes in the entire ecosystem over time and the biological diversity to be able to carry out a successful recovery plan (Witmer 2005). However, non-threatened species should not be neglected in monitoring efforts as their conservation status might change in the future, therefore monitoring of those species is important as well. Many extinctions could have been prevented if the data on population decline had been available at the time and if some kind of conservation action was implemented (Martin et al. 2012; Robinson et al. 2018).

Despite its significance, wildlife monitoring is facing various challenges. One of the major constraints for biodiversity monitoring is the issue of underfunding, often in combination with poorly specified objectives. Such conditions can impede the work of biologists and are exacerbated by pressures from a contractor (e.g., federal/state conservation agency) which may require a lot of information as fast as possible for a minimal price (Robinson et al. 2018). Therefore, objectives and goals, relevant questions, appropriate time schedules, and funding should be agreed upon designing and implementing proper monitoring methods (Witmer 2005; Lindenmayer et al. 2012).

Additionally, the effectiveness of monitoring can be affected by numerous ecological variables and interacting factors (Lindenmayer et al 2012). Wildlife monitoring is particularly challenging in species with low population densities and low detection rates. Some ethical issues further complicate the monitoring of threatened species as their fragile existence restricts the use of certain experimental methods (Robinson et al. 2018). In the case of wolves and other species of large carnivores, the major challenges represent their elusive behavior in combination with low population densities over large and often remote areas (Mumma et al. 2015).

Various methods of wildlife monitoring have been developed to cope with different factors, including direct methods or indirect methods. Direct methods include observations, mark-recapture, transects, while indirect methods involve collection of fecal or hair samples, burrow counts, or other forms of signs of animals in the area (Witmer 2005). However, not all methods are suitable for every species/habitat, thus the choice of methods generally depends on the ecology and habitat of studied species as well as on the available resources. For example, in highly visible species in open habitats (e.g., large ungulates), it is possible to employ direct observations (Zero et al. 2013). On the

other hand, direct methods are not very suitable for species such as large carnivores as their detection are challenging (Proffitt et al. 2015). Large carnivores are naturally very elusive, and centuries of persecution have resulted in even stronger predisposition to avoid humans, therefore indirect methods such as the use of non-invasive genetic samples appear to be a better option for their monitoring (Caniglia et al. 2012).

1.1.1. Non-invasive genetic monitoring

Non-invasive genetic monitoring was developed relatively recently. Non-invasive samples were first employed in 1992 for monitoring brown bears (*Ursus arctos*; Taberlet & Bouvet, 1992) and in chimpanzees (*Pan troglodytes*) to study their social structure (Morin & Woodruff 1992; Carroll et al. 2018). Since its introduction in the 1990s, this method has been used many times and it has proven to be a useful tool for the long-term monitoring of large carnivores (Schwartz et al. 2007).

Non-invasive genetic monitoring can be understood as a combination of multiple techniques (field, laboratory, bioinformatic) to study wild populations without disturbing the animals in their natural habitat (Fabbri et al. 2012). One of the best features of this method is the possibility to identify individuals using molecular markers and obtain genetic data from non-invasive samples without the need to capture, or even without observing studied individuals (Schwartz et al. 2007; Carrol et al. 2018). Overall, non-invasive sampling can be defined as a form of sampling when the animals are unaware of the sampling and, therefore, are unaffected by it, they do not exhibit any form of stress response and do not experience reduction in survival or reproduction (Pauli et al 2010). In genetic monitoring, non-invasive samples are considered those which were left by the animals without interference from the researcher (feces, hairs, urine, feathers, and buccal cells from food; Taberlet et al. 1999; Larson et al. 2020). Additionally, alternative DNA-based detection techniques can be used for monitoring, including environmental DNA (eDNA) or invertebrate-derived DNA (iDNA; Schnell et al. 2015).

In comparison, to traditional non-invasive sampling, the eDNA uses genetic material extracted from the environment where it was left by an unknown individual (Rees et al. 2014; Larson et al. 2020). This method has been therefore increasingly applied in combination with genetic metabarcoding for monitoring rare and elusive species as well as for the detection of invasive species (Schwentner et al. 2021). Especially in

aquatic environments, using eDNA is likely to be more sensitive and less labor intensive in the detection of organisms compared to conventional methods such as visual/acoustic surveys, mark-recapture, etc. (Rees et al. 2014; Baker et al. 2016; Larson et al. 2020; Schwentner et al. 2021). Nonetheless, there are some limitations associated with the use of the eDNA approach, as it is susceptible to both false positive and false negative results. Therefore, it is recommended to employ eDNA approach in combination with complementary methods (Rees et al. 2014; Larson et al. 2020).

Similarly, to the eDNA, it is possible to use iDNA for biodiversity surveillance (Massey et al. 2021). iDNA involves the retrieval of genetic material from the gastrointestinal tract of invertebrates such as leeches, mosquitoes, ticks, flies, or midges (Schnell et al. 2015). Metabarcoding the deoxyribonucleic acid (DNA) obtained from an invertebrate body can be then used as an efficient method of profiling the diversity of vertebrate species, particularly in remote habitats with dense vegetation (Massey et al. 2021).

Non-invasive genetic monitoring offers a wide range of applications including the capability to estimate multiple biological and demographic parameters such as hybridization, occupancy, population size, gene flow and even monitoring responses to selective pressures like overhunting or climate change (Carroll et al. 2018). In wolves and other large carnivores in particular, molecular analyses present an amazing opportunity to study expansions of their home ranges, habitat use, and dispersal patterns and even potential hybridization with domestic dogs in their newly inhabited areas. Knowledge about these processes is then crucial for the management of these species and for implementing further conservation policies (De Groot et al. 2016)

1.1.1.1. Genetic markers

Over the years, multiple genetic markers have been developed for monitoring and better understanding of large carnivores. Initially, mitochondrial DNA (mtDNA) was popular as a genetic marker due to its easy amplification and relatively higher mutation rates compared to nuclear genes (Hurst & Jiggins 2005; De Groot 2016). However, it was later replaced due to its biased view of population history caused by the maternal inheritance of mtDNA (De Groot et al. 2016). Therefore, if the male and female history differed, the mtDNA would reflect only the maternal lineage, rather than the history of

the entire species (Hurst & Jiggins 2005). Nonetheless, mtDNA remains useful for taxonomy and it can provide insights into historical population dynamics (Gao et al. 2020). However, its utility is limited in studies focusing on individual-level events (identity, dispersal, mating systems) or in investigating recent loss of genetic variation (Wan et al. 2004).

Molecular markers offer a great alternative to using phenotypes or physical tags (radio collars, microchips, dyes, etc.) for individual identification. By employing the molecular markers, it is possible to use the generated data to estimate abundance and vital rates such as survival and recruitment (Schwartz et al. 2007).

For the estimation of abundance, genetic monitoring is combined with the capture-mark-recapture (CMR) method which traditionally required capturing the animals, marking the animals (collar, microchip, color, etc.), releasing them, and later re-capturing the animals (Lukacs & Burnham 2004). Nonetheless, with non-invasive CMR it is possible to use samples such as feces, hairs, or feathers to identify individuals based on their own unique genetic codes. Obtaining an already-known genetic code is then considered as recapture. Therefore, non-invasive CMR appears as a great choice, particularly in elusive and potentially dangerous animals such as large carnivores (Schwartz et al. 2007).

Nowadays, most non-invasive genetic monitoring studies employ microsatellites as their molecular markers due to their high polymorphism and easy amplification (Fabbri et al. 2012; De Groot et al. 2016; Roques et al. 2019). Therefore, they serve as very effective markers in genetic monitoring (Bryda & Riley 2008). However, their biggest drawback lies in the incomparability of results between laboratories or the requirement for standardization. Additionally, the occurrence of amplification errors in forms of either allelic dropout or false allele amplification is an issue as well (Fabbri et al. 2012; Forcina et al. 2021). The incomparability between laboratories is especially problematic in Europe because of the large number of countries, several transboundary populations of large carnivores, and differences in conservation policies and monitoring approaches among them (De Groot et al. 2016).

Because of limitations such as allelic dropout, false alleles, and incomparability among laboratories, some studies have started using single nucleotide polymorphisms

(SNPs) as molecular markers instead of microsatellites. SNPs are supposed to be more advanced because of their high data quality, abundance in the entire genome, easy comparison among different laboratories, and overall improved genotyping efficiency (Morin et al. 2004; Roques et al 2019). On the contrary, SNPs have proven to be less suitable for detecting recent bottleneck effects due to their tendency to induce monomorphism with allele removal. Microsatellites appear to be more suitable for detecting population expansions over the short term as the SNPs require more time for the new mutations to accumulate. Although, this issue can be resolved by employing numerous loosely connected SNPs (Morin et al. 2004). While the risk of amplification errors is lower in SNPs compared to microsatellites, a low number of SNPs is likely to be less informative for population genetics compared to the microsatellites (Fabbri et al. 2012) as SNPs often require two to six times more markers to achieve the same resolution (Morin et al. 2004). Nonetheless, probably the biggest limitation of SNPs for implementing in non-invasive monitoring is their requirement of high-quality and quantity DNA (Morin & McCarthy 2007).

1.2. Monitoring of Wolves

The importance of monitoring wolves (*Canis lupus*) and other large carnivore species has been increasing as these species are returning to areas they have not occupied for generations. However, the return of large carnivores brings back conflict with humans as they tend to prey on poorly guarded livestock (Echegaray & Vila 2009). On the other hand, large carnivores have cascading effects on lower trophic levels, thus they are considered as 'keystone species' for their ecosystems (Linnel et al. 2000).

Therefore, governments and other institutions around the world invest substantial resources in the forms of monetary compensations, damage prevention, and population monitoring of large carnivores (Echegaray & Vila 2009; Ausband et al. 2014). The public is generally very interested in these species as well. Therefore, large carnivores have the potential to serve as umbrella species for the conservation of the entire ecosystems they occupy, thus protecting their habitat and other species living in it (Linnel et al. 2000).

Yet, monitoring of wolves and other large carnivores can be often challenging both in terms of logistics and finances as they are very elusive and occupy large areas at

low densities (Ausband et al. 2014; Mumma et al. 2015). The most used approaches for monitoring of wolves include methods such as sign surveys (snow tracking), howling and radio/camera tracking, questionnaires distributed among local people, and recently also the use of non-invasive sampling (Llaneza et al. 2014).

Monitoring of wolves in Europe is even more problematic compared to other parts of the world because most of the populations are transboundary, therefore managed by different administrations in each country which often differ politically, economically, and in monitoring practices (Marucco & Boitani 2012). The variety of wolf habitats further complicates this issue, as certain methods might be well-suited to specific regions, but not viable in other areas (e.g., snow tracking; Ausband et al. 2014). Therefore, achieving consistent monitoring intensity across the entire home range is almost impossible (Reinhardt et al. 2015). Large home ranges of the species also limit the employment of camera traps at large scales as it would be very expensive and logistically demanding (Ausband et al. 2014). Additionally, the presence of snow is important for many methods as they rely on the presence of signs found in the snow (Blanco & Cortés 2012). Therefore, using a combination of multiple methods is usually recommended to get more precise results, even though it significantly increases financial expenses (Ausband et al. 2014).

Nonetheless, the territoriality and behavioral traits of wolves make them well-suited for applying non-invasive genetic monitoring in combination with other methods such as snow tracking or telemetry. Wolves often leave signs (scats) in prominent places along roads and trails, making them particularly suitable for data collection (Stenglein et al. 2010). Compared to other methods, genetic monitoring does not strictly rely on the presence of snow. Although the presence of snow and low temperatures are beneficial for the collection of non-invasive samples (Galaverni et al. 2012) as presence signs are more visible and DNA degradation happens much more slowly. Rapid DNA degradation in warmer temperatures can lead to low amplification rates and genotyping errors possibly creating false genotypes, consequently leading to overestimations of true population sizes (Fabbri et al. 2012). Therefore, sampling should be ideally carried out during winter (Agetsuma-Yanagihara et al. 2017).

In Europe, the combination of snow tracking and collecting non-invasive samples for genetic analyses have been increasingly used in multiple countries (Blanco & Cortés

2012) emerging as a standard method for monitoring large carnivores across the continent (Reinhardt et al. 2015). However, in the United States, telemetry combined with aerial surveys remains the most common method (Blanco & Cortés 2012). However, the growing numbers of wolves in the United States make telemetry less efficient as it is getting more difficult to maintain a high percentage of collared individuals within the population (Stenglein et al. 2010).

1.3. Wolf distribution in Europe

Wolves were once widespread across Europe. However, their numbers were decimated during the last two centuries due to habitat loss, depletion of their natural prey, and persecution from humans. This conflict resulted in a global reduction of the wolf's range to 68% and led to the extinction of wolves and other large carnivores in most parts of Western and Central Europe. It also caused a significant fragmentation and reduction of the remaining populations across the continent (Jedrzejewski et al. 2004; Hindrikson et al. 2017; Hulva et al. 2018; Silva et al. 2020).

Nonetheless, wolves have been experiencing a gradual expansion since the second half of the 20th century mainly due to the implementation of conservation measures and changes in land use practices (Hindrikson et al. 2017; Silva et al. 2020). This has led to range expansions as well as the reconnection of historically isolated populations (Szewczyk et al. 2021). The combination of conservation measures with long-distance dispersal and high reproductive output of wolves enabled the successful recolonization of their historical ranges even within highly human-dominated areas across Europe (Fig. 1; Hindrikson et al. 2017; Gula et al. 2020).

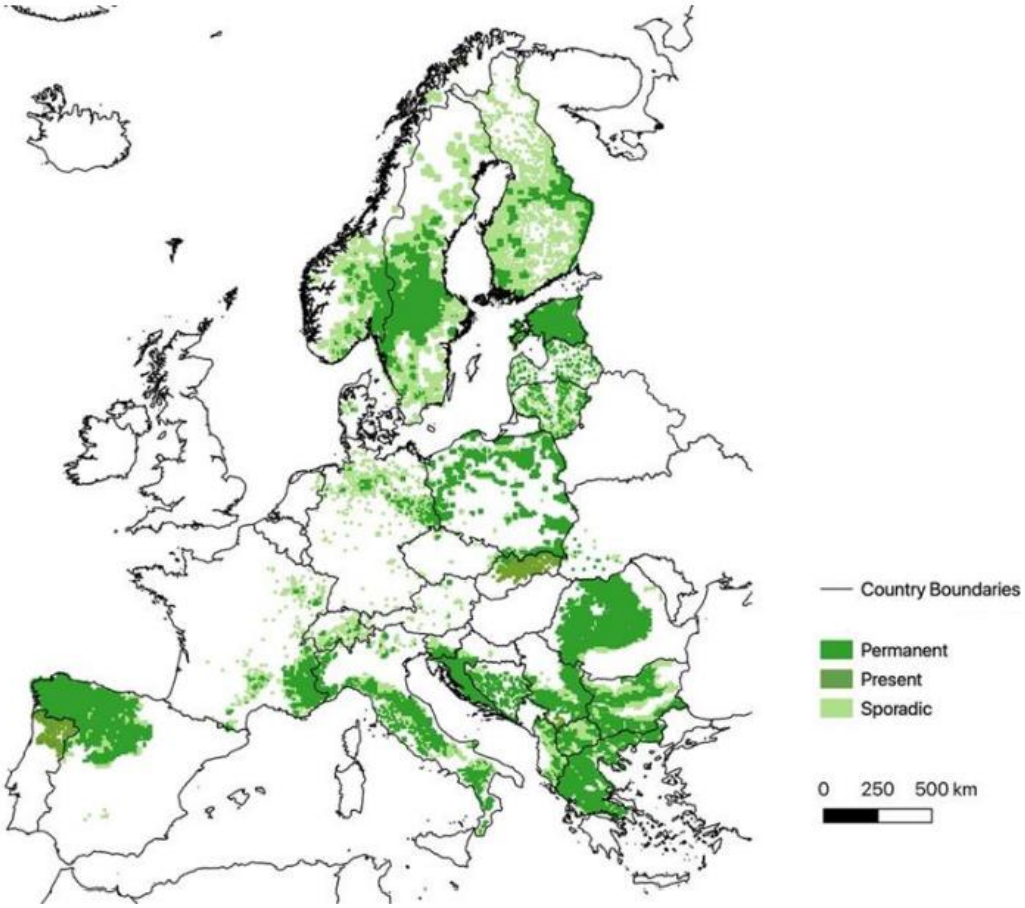

Figure 1: Distribution of wolves (*Canis lupus*) in Europe as of 2016 (Boitani et al. 2022)

According to the latest data from the International Union for Conservation of Nature (IUCN), the number of wolves in Europe (excluding Russia) has been estimated to be more than 17,000 individuals (IUCN 2018), making them the second most abundant large carnivore species on the continent after the brown bear (*Ursus arctos*; Chapron et al. 2014). Wolf range is estimated to be 800,000 km^2 spanning across 28 European countries, and this range continues to expand (Gula et al. 2020). Nowadays, wolves are present in nearly all countries of continental Europe and they are categorized into 10 distinct populations (Fig. 2), based on their history, distribution, social, genetic, and ecological factors: Alpine, Baltic, Carpathian, Central European, Dinaric-Balkan, Italian

Peninsula, Karelian, North Western Iberian, Scandinavian and Sierra Morena (Table. 1; Boitani et al. 2015).

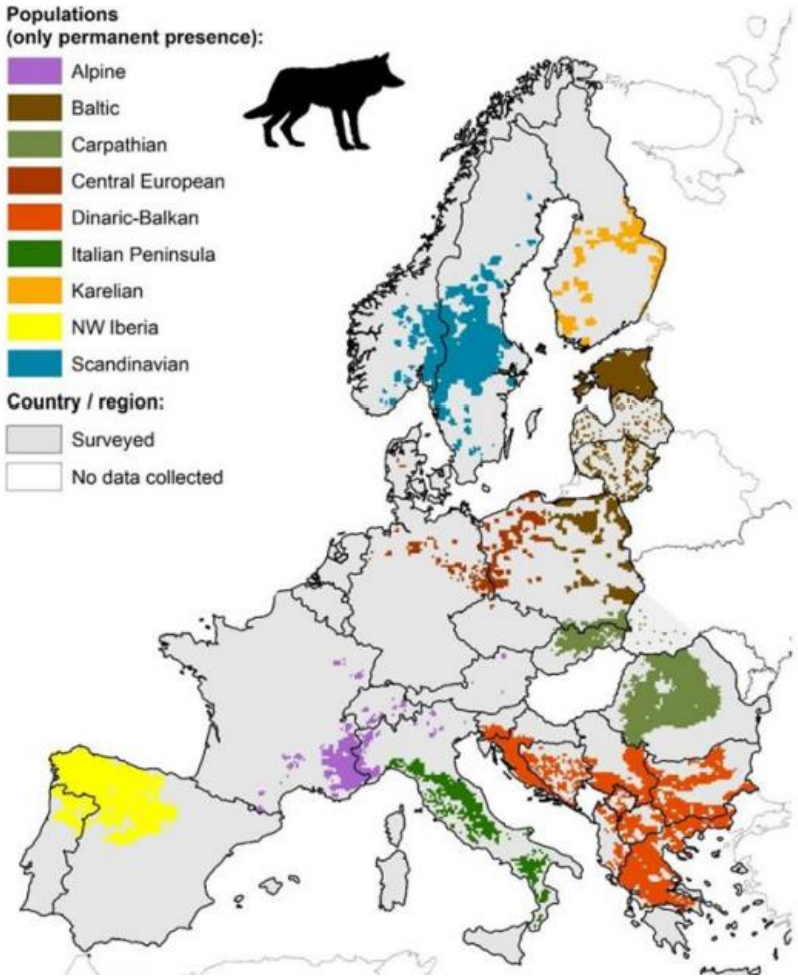

Figure 2: Wolf populations in Europe as of 2016 (Boitani et al. 2022)

Table 1: List of wolf populations in Europe (IUCN 2018; Boitani et al. 2022; EC 2022)

Population	Countries	Size	Trend
Alpine	Italy, France, Switzerland, Austria, Slovenia	822 – 1099	Increasing
Baltic	Estonia, Latvia, Lithuania, Poland	2190 – 2790	Stable

Carpathian	Slovakia, Czech Republic, Poland, Romania, Serbia	3900 – 4700	Stable
Central European Lowlands	Germany, Poland, Czech Republic	c.1850	Increasing
Dinaric-Balkan	Slovenia, Croatia, Bosnia & Herzegovina, Montenegro, Northern Macedonia, Albania, Serbia (incl. Kosovo), Greece, Bulgaria	c. 5000 – 5500	Unknown
Italian Peninsula	Italy	2020 – 2645	Slightly increasing
Karelian	Finland	275 – 315	Stable to increase
NW Iberian	Spain, Portugal	2,500	Stable
Scandinavian	Norway, Sweden	c. 550	Increasing
Sierra Morena	Spain	0	Extinct

1.3.1. Wolf populations in Central Europe

With the expansion of wolves throughout Europe, previously isolated populations have started to reconnect (Szewczyk et al 2022). Central Europe due to its position is considered a “crossroad” or genetic junction and thus serves as a contact zone for several genetically distinct wolf populations (Hulva et al. 2018; Fig.3).

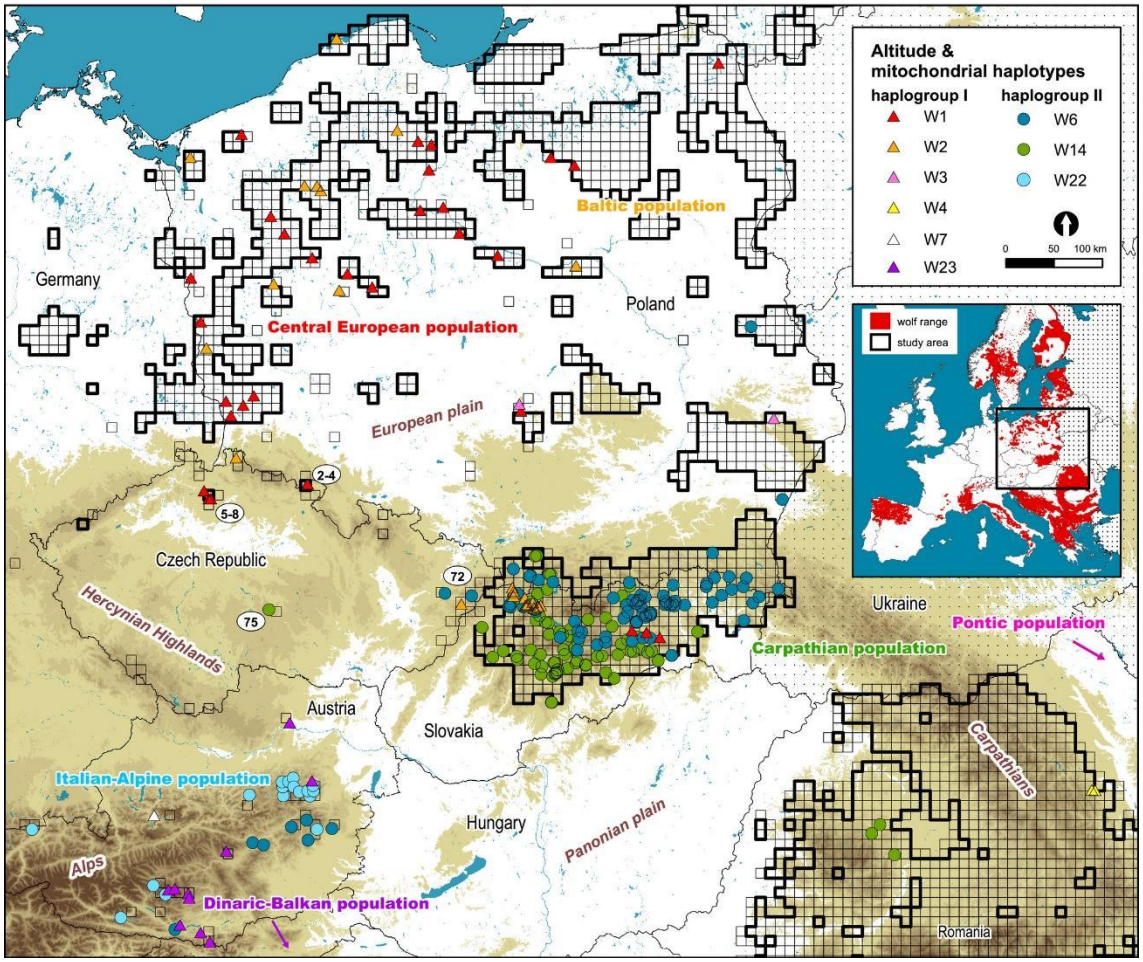

Figure 3: Populations of wolves occurring in Central Europe (Hulva et al. 2018).

1.3.1.1. Alpine population

Wolves have been recolonizing the Alps since the 1990s when individuals from the Apennines first reached the southwestern Alps in 1992 (Lucchini et al. 2002). Today, the Alpine population inhabits the entire Alpine arc across 7 countries with 206 packs and 37 pairs and a total number of 243 reproductive units across the area (Wolf Alpine Group 2022). This population was most likely founded by multiple genetically unrelated individuals from the Apennine population with an estimated number of effective founders to be 8-16 individuals. As a result of strong bottleneck and founder effect during the

recolonization, the Alpine population shows significantly lower level of genetic diversity (in terms of heterozygosity, allelic richness, and number of private alleles) compared to its source population in the Apennines (Fabbri et al. 2007), which is already one of the least genetically diverse populations in Europe (Hindrikson et al. 2017). Although the two populations still maintain some degree of genetic and demographic connection, they strongly differ in ecological and socio-economic contexts. Therefore, even though they are still moderately connected genetically (Fabbri et al. 2007), the Alpine population is already functionally autonomous (Wolf Alpine Group 2014; Wolf Alpine Group 2022). A similar recolonization trend has been recently observed in the eastern Alps from the Dinaric-Balkan population which might potentially improve the genetic diversity of the Alpine population (Fabbri et al. 2014).

1.3.1.2. Carpathian population

The Carpathian population inhabits a large and relatively continuous area within and around the Carpathian arc connecting a large population in the east with a relatively small population in the west. The western population has experienced a severe bottleneck effect in the past which resulted in genetic differentiation (Fig.3; Pilot et al. 2014). The majority of this population can be found in the eastern part, in Romania and Ukraine (2300-2700 individuals), then also in Slovakia (340-450 individuals), and Poland (250-300 individuals) with smaller numbers found in Czechia, Hungary, and Serbia (Hindrikson et al. 2017; Fehér at al. 2022). The Carpathian Mountains also represent one of the largest wolf refugia in Europe, therefore this area is particularly important for the long-term survival of this species in this region as well as its potential to serve as a link between populations in Northern and Southern Europe (Gula et al. 2009).

The Carpathian population is genetically distinct from its neighboring populations, and it is carrying unique genetic features such as mitochondrial DNA typical for wolves from the Ice Age and distinct prey composition (Jędrzejewski et al. 2012; Hulva et al. 2018). Further, wolves from the Carpathian population differ morphologically as their skull size, particularly among males, is generally larger compared to individuals from Lowland and Dinaric-Balkan populations (Milenković et al. 2010; Ericson et al. 2020).

1.3.1.3. Central European population

The Central European (CE) population consists mainly of individuals originally inhabiting the Northern European Plain in Western Poland and Eastern Germany (Hindrikson et al. 2017; Schley et al. 2021). This population was probably founded in the 1990s by a small group of individuals from north-eastern Poland originating from the Baltic population (Czarnomska et al. 2013; Hindrikson et al. 2017). Even though the CE population and the Baltic population represent the same phylogeographic lineage, they are demographically independent from each other, and their allele frequencies differ as well. This has been probably caused by the lethal management in the Baltic states which reduces gene flow between the two populations, thus it is not strong enough to reduce the founder effect in the CE population (Szewczyk et al. 2021). However, some scientists argue that those two populations should be considered as one with continuous range, habitat characteristics, management regimes, and genetic structure with the only difference in stage of recovery (the CE population is in the earlier stage of recovery), thus there is no need for an additional management zone (Gula et al. 2020).

Compared to the Carpathian population, the CE population shows a much stronger expansion trend due to its better adaptability in densely populated landscapes (Hulva et al. 2018). Because of the adaptability and more suitable habitat, the CE population has been probably the fastest growing population in Europe with packs already established in countries such as Austria, Belgium, Czechia, Denmark, Luxembourg, and the Netherlands and it is now estimated to consist of approximately 1,850 individuals (Reinhardt et al. 2015; Schley et al. 2021, Boitani et al. 2022). However, the genetic diversity of the population in newly colonized areas is generally lower compared to Carpathian and Balkan-Dinaric populations most likely due to strong founder effect (Hindrikson et al. 2017; Hulva et al. 2018; Szewczyk et al. 2019).

1.3.1.4. Dinaric-Balkan population

The Dinaric-Balkan population spreads across the Dinaric-Balkan Mountain range across several countries from Slovenia to Greece (Hindrikson et al. 2017). This region was one of the glacial refugia in Europe during the Last Glacial Maximum, therefore it still retains a substantial level of historic genetic diversity in many species (Randi et al. 2000; Gomerčić et al. 2010). For this reason, the Dinaric-Balkan population

has potential to serve as a source of genetic diversity for its neighboring Apennine and Alpine populations as well as a bridge between populations in the West and in the East (Djan et al. 2014; Šnjegota et al. 2021). Similar to the Carpathians, this region was also less exposed to human activities during the 20th century, therefore the populations of wolves and other species stayed quite stable compared to other regions in Europe and therefore they preserved a significant level of genetic diversity (Gula et al. 2009; Ražen et al. 2016; Šnjegota et al. 2023).

Nonetheless, even though the Dinaric-Balkan population represents one of the biggest wolf populations in Europe with a significant level of genetic diversity, the quality of data can be quite poor for some countries, especially in the southern regions due to multiple different management plans among the countries across the Balkan peninsula and lack of monitoring efforts in some of the countries (Boitani et al. 2022).

1.3.2. Wolves in the Czech Republic

Wolves were a common species in the area of modern Czech Republic until the end of the 17th century, however, their numbers started to rapidly decrease in the 18th as a result of persecutions. Wolves were considered harmful and extremely dangerous, therefore the official goal was a complete eradication of this species (Bufka et al. 2005). In Bohemia, the original Central European wolf population was most likely exterminated by the end of the 18th century. In Moravia and Silesia, wolves managed to survive until the early 20th century, probably caused by the proximity to the Carpathian population. After the eradication, there were only sporadic occurrences of usually single individuals incoming from neighboring countries (Austria, Poland, Slovakia). Those individuals were usually hunted and killed shortly after being sighted (Adreska 2013; Black Wolf 2023).

Nonetheless, in the 1990s, wolves made their first reappearance in the Beskydy Mountains, and they have maintained a regular presence there since. These individuals were immigrants from the Carpathian population, however, the first reproduction was not recorded until 2019 as their expansion in this area has been limited by poaching as well as by legal hunting in neighboring Slovakia (Dolejský 2021; Šelmy 2023).

Since the early 2010s wolves have also started to expand to the northern parts of the Czech Republic from Poland and Germany (Central European population; AOPK 2023). The first reproducing pack was established in 2014 in the Ralsko region and has

been reproducing annually until 2020. Expansions to other regions followed quickly after and since 2015, wolves have started to be regularly observed in most of the mountain ranges near the northern borders of the Czech Republic (Giant Mts., Ore Mts., Jizera Mts, Bohemian Switzerland, Jeseníky Mts., etc.; Dolejský 2021; Šelmy.cz 2023). Expansion to the southern parts of Czechia started few years later with the first reproducing pack being documented in 2017 in the Bohemian Forest Mts. This pack was interesting because it was established by individuals from two different populations, the male originated from the Alpine population, while the female belonged to the Carpathian population (AOPK 2023).

Overall, wolves have predominantly inhabited areas near the borders (Fig. 4), however, occasional occurrences have been observed throughout Czechia (AOPK 2023, Šelmy.cz 2023). The latest data from 2022 show that during the years 2020/2021 there were 24 wolf territories that were extended, at least partially, into the Czech Republic. Out of these, eighteen were occupied by packs, four by pairs, and two by individual animals (Carnivores.cz 2022; Fig.4).

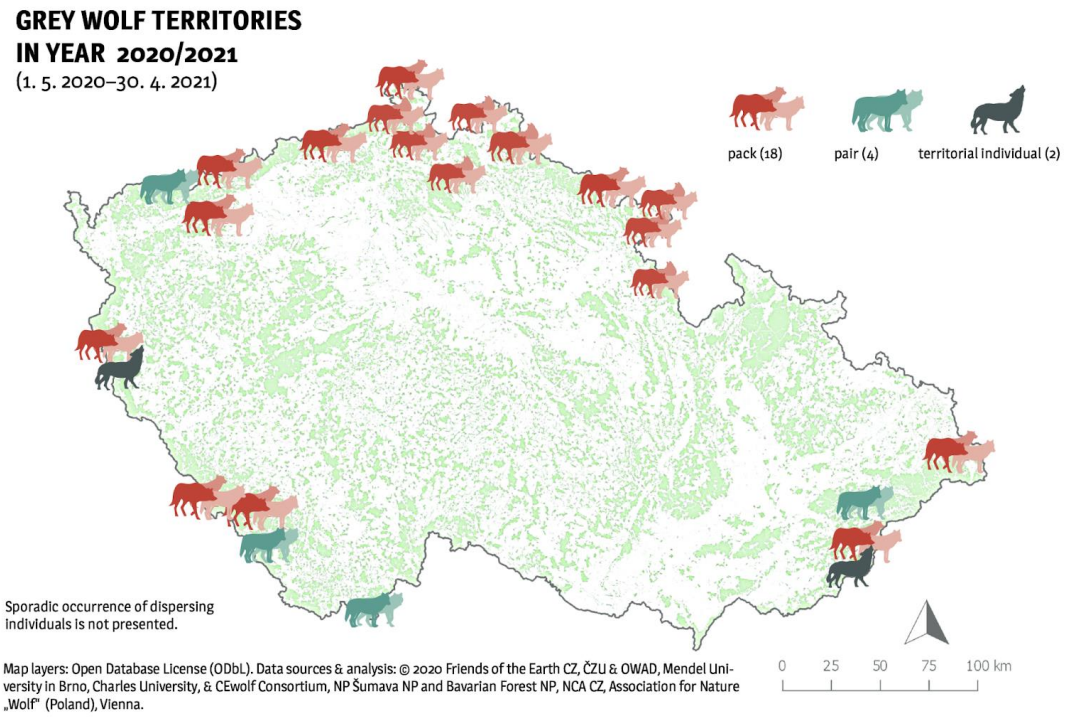

Figure 4: A map of the Czech Republic depicting wolf territories in the year 2020/2021 (Carnivores.cz 2022)

Currently, the most numerous wolf population is the Central European population followed by the Carpathian population (Dolejský 2021). However, there have also been recordings of individuals from the Alpine population and even individuals from the Dinaric-Balkan population in southern Bohemia (OSU 2018; Hulva et al. 2018).

The expansion of wolves is a dynamic process, and the number of wolf territories is steadily increasing every year (AOPK 2023). However, the growing number of wolves also brings the potential for increase in livestock predation, resulting in the escalation of human-wildlife conflicts and the associated challenges (economic losses, compensations, negative perception, etc.). Thus, the significance of carnivore monitoring is on the rise as well.

2. Aims of the Thesis

- Genetic monitoring of grey wolf (Canis *lupus)* in selected areas of Central Europe during years 2020 – 2023
- To confirm species status and test hybridization using genetic tools
- To determine the population origin of sampled individuals

3. Methodology

3.1. Sampling

DNA samples were provided by a non-governmental organization (NGO) Friends of the Earth Czech Republic (Hnutí Duha), one of the most prominent and active environmentalist organizations in the Czech Republic (Fagan & Jehlička 2003). The collection of samples was conducted by groups called "Wolf Patrols". Those are groups of volunteers trained by the Friends of the Earth Czech Republic and their primary goal is to conduct continuous monitoring in areas with the presence of large carnivores (gray wolf, European lynx, brown bear) and wild cats. Their aim is to discover occurrence signs of these species such as feces, hairs, tracks, or prey carcasses while also discouraging potential poaching activities in those areas (Friends of the Earth Czech Republic 2016; Friends of the Earth Czech Republic 2023). This effort was part of the project called Coexistence with Large Carnivores (Soužití s velkými šelmami).

DNA samples were collected and stored either in 50 ml tubes with 96% ethanol or in the case of hair samples, those could also be stored in envelopes with silica gel. All types of samples were later stored at -20°C. Each individual sample had to be labeled with its GPS coordinates, date, name of the person who found it, unique code of donor, and expected species. Later, those samples were transported in thermal boxes to laboratories at the Czech University of Life Sciences Prague or Charles University for further analyses.

3.2. Laboratory procedures

Laboratory procedures were unified over the two laboratories. Therefore, the sample processing could be delegated to both institutions. The Czech University of Life Sciences Prague conducted the analysis for the majority of samples (459), with contributions from the author and other members of the MEGERA research group. The remaining samples (130) were analyzed by students at Charles University. The author of this thesis was responsible for all the subsequent bioinformatic analyses and graphical representations.

3.3. DNA isolation

Extraction of DNA from samples was performed using commercial kits developed by companies *Qiagen*, *Macherey-Nagel*, or *Geneaid*. These types of extraction kits have gained popularity among molecular ecologists due to their fast and easy DNA purification and decreased risk of cross-sample contamination (Eggert, Maldonado, Fleischer 2005).

QIAamp Fast Stool Mini Kit (Qiagen) and *Nucleo-spin® DNA Stool (Macherey-Nagel)* were used for fecal samples. Samples had to be first taken out of the tubes where they had been stored and put on filtration paper in a flow box. Once samples were taken out of the tubes, a small amount of the external part of the fecal sample, which is most likely to contain epithelial cells from the digestive tract (thus the DNA of the individual) was cut off and left for a few minutes for evaporation of ethanol residues. Once the sample was ready for further processing, DNA extraction was carried out according to the protocols provided by the manufacturer.

DNeasy Blood & Tissue Kit (Qiagen) and *Tissue Genomic DNA Mini Kit (Geneaid)* were used for samples of blood, tissue, hair, and swabs. In hair samples, only the follicles of the hairs were used for the analysis because they contain a majority of hair cellular DNA (Graziano et al. 2016).

QIAamp Fast Stool Mini Kit (Qiagen) was used for the isolation of DNA from urine samples, with modified first step. The snow-urine mixture had to be defrosted and after that, 15 ml of the snow-urine mixture, 33 ml of ethanol (96%), and 1,5 ml of 3M sodium were poured into a 50 ml tube and left overnight at a temperature of -20°C. Following morning, the sample was centrifuged for 60 minutes at -20°C at speed of 5,500 rcf (relative centrifugal force; Hausknecht et al. 2006). The sedimented pellet at the bottom of the tube was then used for extraction according to the manufacturer's protocol for the kit.

Once the purification of DNA was complete, the purity and concentration of DNA in each sample were measured using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher).

3.4. Amplification of genetic markers

Nuclear microsatellites were used as genetic markers for species, individual identification, and population analyses. A Set of 20 microsatellite loci and the amelogenin for sex determination were selected based on a combination of loci used in previous studies focusing on genetic monitoring of wolves in the Czech Republic (Hulva et al. 2018; Báčová 2019) and loci that are currently being used by laboratories within the CEwolf consortium.

Fluorescently labeled primer pairs were divided into two multiplex mixtures. The third mixture was later created as a combination of the primers used in Multiplex 1 and Multiplex 2 with lower success rates for additional comparison and overall improvement of the quality of amplification. Additional information about genetic markers and multiplex mixtures can be found in Table 2.

Table 2: List of used genetic markers.

Multiplex 1						
Locus name	Forward sequence	Reverse sequence	Dye	Size (bp)	Repetition	Source
FH2088	CCCTCTGCCTACATCTCTGC	TAGGGCATGCATATAACCAGC	6-FAM	93-129	Tetra	Francisco et al. (1996)
FH2054	GCCTTATTCATTGCAGTTAGGG	ATGCTGAGTTTTGAACTTCCC	6-FAM	136-172	Tetra	Cho (2005)
FH2087	CTGCCACATTCACTGATGC	CAACTCCCTCCCTCATTTCA	6-FAM	224-252	Tetra	Francisco et al. (1996)
PEZ17	CTAAGGGACTGAACTTCTCC	GTGGAACCTGCTTAAGATTC	VIC	220-240	Tetra	Cho (2005)
CXX279	TGCTCAATGAAATAAGCCAGG	GGCGACCTTCATTCTCTG AC	NED	109-133	Di	Ostrander et al. (1993)
REN169O18	CACCCAACCTGTCTGTTCCT	ACTGTGTGAGCCAATCCCTT	NED	154-170	Di	Guyon et al. (2003)

FH2097	CAATGTCGAATTCCATGGTG	ATGGAGCAAGATGTGTTTGTG	NED	260-305	Tetra	Francisco et al. (1996)
INRA21	ATGTAGTTGAGATTTCTCCTACGG	TAATGGCTGATTTATTTGGTGG	PET	87-111	Di	Mariat et. al. (1996)
FH2001	TCCTCCTCTTCTTTCCATTGG	TGAACAGAGTTAAGGATAGACACG	PET	132-156	Tetra	Francisco et al, (1996)
REN169D01	AGTGGGTTTGCAAGTGGAAC	AATAGCACATCTTCCCCACG	PET	199-221	Di	Guyon et. al. (2003)
Multiplex 2						
Locus name	Forward sequence	Reverse sequence	Dye	Size (bp)	Repetition	Source
FH2096	CCGTCTAAGAGCCTCCCAG	GACAAGGTTTCCTGGTTCCA	6-FAM	96-100	Tetra	Francisco et al 1996
FH2137	GCAGTCCCTTATTCCAACATG	CCCCAAGTTTTGCATCTGTT	6-FAM	153-165	Tetra	Francisco et al 1996
INU055	CCAGGCGTCCCTATCCATCT	GCACCACTTTGGGCTCCTTC	6-FAM	190-216	Di	Ichikawa et al. (2002)
AHTK211	TTAGCAGCCGAGAAATACGC	ATTCGCCCGACTTTGGCA	VIC	83-101	Di	Thomas et al, 1997
VWF	CTCCCCTTCTCTACCTCCACCTCTAA	CAGAGGTCAGCAAGGGTACTATTGTG	VIC	118-178	Hexa	Shibuya et al. (1994)
FH2161	TCAGCAAGAAACCCTCCAGT	TGTTAGATGATGCCTTCCTTCT	VIC	219-248	Tetra	Francisco et al 1996
FH2140	AAATGGAACAGTTGAGCATGC	TGACCCTCTGGCATCTAGGA	NED	99-149	Tetra	Francisco et al 1996
Amelogenin	GTGCCAGCTCAGCAGCCCGTGGT	TCGGAGGCAGAGGTGGCTGTGGC	NED	180;216		Chen et al. (1999)
CPH5	TCCATAACAAGACCCCAAAC	GGAGGTAGGGGTCAAAAGTT	PET	111-119	Di	Fredholm & Wintero (1995)
REN64E19	TGGAGAGATGATATCCAAAAGGA	ATTCGCCCGACTTTGGCA	PET	139-155	Di	Breen et al. (2004)
FH2010	AAATGGAACAGTTGAGCATGC	CCCCTTACAGCTTCATTTTCC	PET	217-233	Tetra	Cho (2005)
Multiplex 3						
Locus name	Forward sequence	Reverse sequence	Dye	Size (bp)	Repetition	Source
FH2096	CCGTCTAAGAGCCTCCCAG	GACAAGGTTTCCTGGTTCCA	6-FAM	96-100	Tetra	Francisco et al 1996
FH2137	GCAGTCCCTTATTCCAACATG	CCCCAAGTTTTGCATCTGTT	6-FAM	153-165	Tetra	Francisco et al 1996

FH2087	CTGCCACATTCACTGATGC	CAACTCCCTCCCTCATTTCA	6-FAM	224-252	Tetra	Francisco et al. (1996)
VWF	CTCCCCTTCTCTACCTCCACCTCTAA	CAGAGGTCAGCAAGGGTACTATTGTG	VIC	118-178	Hexa	Shibuya et al. (1994)
FH2161	TCAGCAAGAAACCCTCCAGT	TGTTAGATGATGCCTTCCTTCT	VIC	219-248	Tetra	Francisco et al 1996
FH2017	AGCCTCTATAATCACGTGAGCC	CCCAGTACCACCTTCAGGAA	VIC	260-276	Tetra	Francisco et al. (1996)
FH2140	AAATGGAACAGTTGAGCATGC	TGACCCTCTGGCATCTAGGA	NED	99-149	Tetra	Francisco et al 1996
FH2001	TCCTCCTCTTCTTTCCATTGG	TGAACAGAGTTAAGGATAGACACG	PET	132-156	Tetra	Francisco et al, (1996)
FH2010	AAATGGAACAGTTGAGCATGC	CCCCTTACAGCTTCATTTTCC	PET	217-233	Tetra	Cho (2005)

Polymerase chain reaction (PCR) was performed in a total volume of 10 µl of the following mixture: 5 µl of mastermix from *Multiplex PCR plus kit (Qiagen)*, 3 µl of PCR-free H_2O, 1 µl of primermix and 1 µl of isolated DNA. Thermal cycler T100 ™ (*Bio Rad)* was used to run the PCR reaction using the following protocol:

1. 95 °C, 5:00
2. 95 °C, 0:30
3. 60 °C, 1:30
4. 72 °C, 0:30
5. Go back to Step 2, 34x
6. 68 °C, 20:00
7. 12 °C, ∞

PCR products were later used for fragmentation analysis which was conducted in a total volume of 10 µl of the following mixture: 8,75 µl of Formamide, 0,25 µl of GeneScan™ 500 LIZ™ dye Size Standard (*Thermo Fisher*), and 1 µl of the PCR product. The mixture was incubated for 5 minutes in Thermal cycler T100 ™ (*Bio Rad)* at 95 °C. The fragmentation analysis itself was conducted in the genetic sequencer ABI Prism 3100 Avant Genetic Analyzer (*Applied Biosystems*) using polymer POP4 and standard DS-33 in the sequencing laboratory at Charles University.

3.5. Genotyping

Allele scoring of electropherograms obtained from fragmentation analysis was analyzed using software GENEIOUS PRIME (Kearse et al. 2012). Sizes of each fragment of amplified DNA were determined by comparison to the internal size standard GeneScan™ 500 LIZ™ dye Size Standard (*Thermo Fisher*). The intensity of the peaks shown in the GENEIOUS PRIME software corresponds to the amount of amplified DNA in the sample (ATF 2018).

To obtain reliable genotypes, the multiple-tube approach was used (Navidi et al. 1992). It consists of repetitions of experiments (PCR, fragmentation analysis, and genotyping) multiple times using the same DNA sample to compensate for the genotyping errors. Such errors can be allelic dropout, which produces false homozygotes, or misinterpretation of amplification artifacts as alleles which creates a false heterozygote (Tamberlet et al. 1999). Genotypes were therefore deducted from the entire set of experiments. Once the genotypes were reliable, the allelic table from GENEIOUS PRIME was exported into a sheet in MS Excel (Microsoft Corporation 2018).

For non-invasive samples (feces, hairs, swabs, urine), the first experiment was conducted using only Multiplex 1 and Multiplex 2. In the case that the amplification rate was very low (<30% in both primer mixes), those samples were not repeated, and they were not used for further analyses. Samples with higher amplification rates were repeated using all three primer mixes until it was possible to obtain reliable genotypes. The number of repetitions depended on matches and mismatches of the genotypes (from 2 up to 5 and even more in some cases).

Samples of blood and tissue usually didn't require more repetitions as the quality of DNA is expected to be much higher than in the non-invasive samples, thus they do not require as many repetitions (Ghatak et al. 2013; Ferreira et al. 2018). For this reason, we used all three primer mixes already during the first experiment in samples of blood and tissue and repeated the experiment only if additional reassurance was required.

3.6. Species determination

The Bayesian Clustering inference in software STRUCTURE 2.3.4. (Pritchard et al. 2000) was used to assign each genotype to the respective species. The software assigns genotypes to specific clusters (K) based on their allelic frequencies and estimates the *Individual Q-matrix* (Qi) which represents the membership coefficient of each individual to the respective cluster (Porras-Hurtado et al. 2013). Datasets containing genotypes of foxes (*Vulpes vulpes*), jackals (*Canis aureus*), domestic dogs (*Canis familiaris*; pure breed and feral), and wolves from the Carpathian and Central European populations were used as references.

Burn-in period was set to 200,000 Markov Chain Monte Carlo (MCMC) iterations and the after-burn-in period was set to 800,000 MCMC iterations with the number of clusters set from K=1 to K=7, each repeated three times. Software STRUCTURE SELECTOR (Li & Liu 2018) was later used to select and visualize the optimal number of clusters. Genotypes not corresponding to wolf clusters were then omitted from population genetic analyses.

3.7. Identity analysis

Identity analysis was conducted in the software CERVUS (Kalinowski et al. 2007), which can identify individuals even if the genotypes do not match fully. It compares genotypes using the likelihood approach. A minimum of 7 matching loci and a maximum of 5 mismatches were allowed for the identity analysis. Obtained results (matching genotypes) had to be checked in the GENEIOUS PRIME software together with their GPS coordinates. Genotypes that proved to be identical were considered as recaptures. Duplicates of already known genotypes were not used for further population genetic analyses.

3.8. Population origin

The population origin was determined using Bayesian clustering analysis in software STRUCTURE 2.3.4. (Pritchard et al. 2000). The settings of the analyses were the same as in the case of species determination. However, comparative datasets

comprised three populations of wolves with known origins and one dataset of domestic dogs. These populations (Alpine, Carpathian, and Central European) were selected due to their potential occurrence in the Czech Republic. Due to the limited number of loci in the comparative dataset of the Alpine population (12 loci), the Bayesian Clustering analysis was conducted twice: initially using 20 loci, followed by a second analysis using 12 loci. Software STRUCTURE SELECTOR (Li & Liu 2018) was again used to select the ideal number of clusters and to better visualize our results.

Principal Coordinate Analysis (PCoA) was performed using GenAlEx 6.5 software (Peakall & Smouse 2012) within Microsoft Excel to visualize similarities between the analyzed genotypes and comparative populations. Furthermore, GenAlEx 6.5 was used to calculate genetic parameters such as heterozygosity, coefficient of inbreeding, fixation index, and the number of private alleles of each population. Further, Software Genepop 4.7.5. (Raymond & Rousset 1995) was utilized assess conformity with the Hardy-Weinberg equilibrium (HWE). To estimate number of null alleles, software Micro-Checker (Oosterhout et al. 2004) was employed.

Additionally, NewHybrids software (Anderson & Thompson 2002) was utilized to detect admixed individuals in our dataset. Samples were divided based on population affiliation determined by the STRUCTURE analysis and dataset of domestic dogs was used for comparison. The Burn-In period was set to 200,000 MCMC iterations and the after-burn-in period was set to 5,000,000 MCMC iterations.

4. Results

4.1. Sampling

Samples were collected between January 2020 and April 2023. The total number of collected samples was 645, however, some samples could not be used due to insufficient information about their collection or a very bad condition, thus those samples had to be removed before the analysis. Therefore, the total number of analyzed samples was 589.

Most of the samples consisted of non-invasive samples with the majority being fecal samples (516), followed by a significantly smaller number of hair samples (25), urine (24), swabs from prey carcasses (9), mixtures of blood and urine (5) and vomit (2). Additionally, several invasive samples such as blood (7) and tissue (1) from injured animals and road kills were also included in the study (Fig. 5). Locations of individual sampling occasions divided based on the type of samples are depicted in Figure 6.

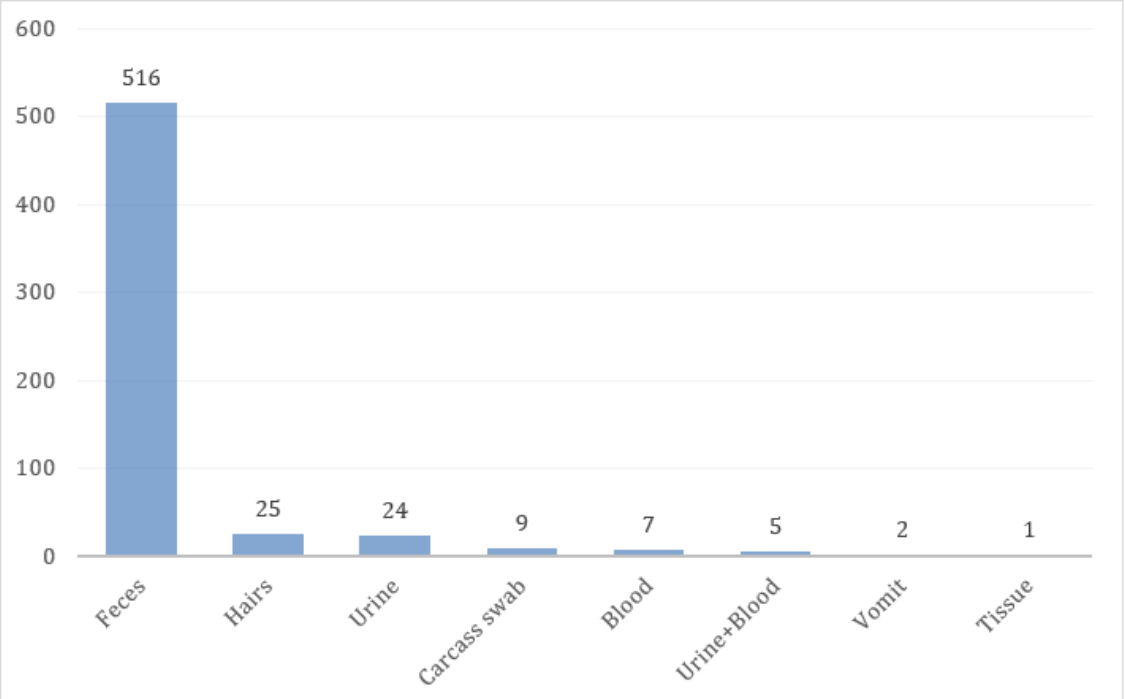

Figure 5: Number of each type of sample collected for the study.

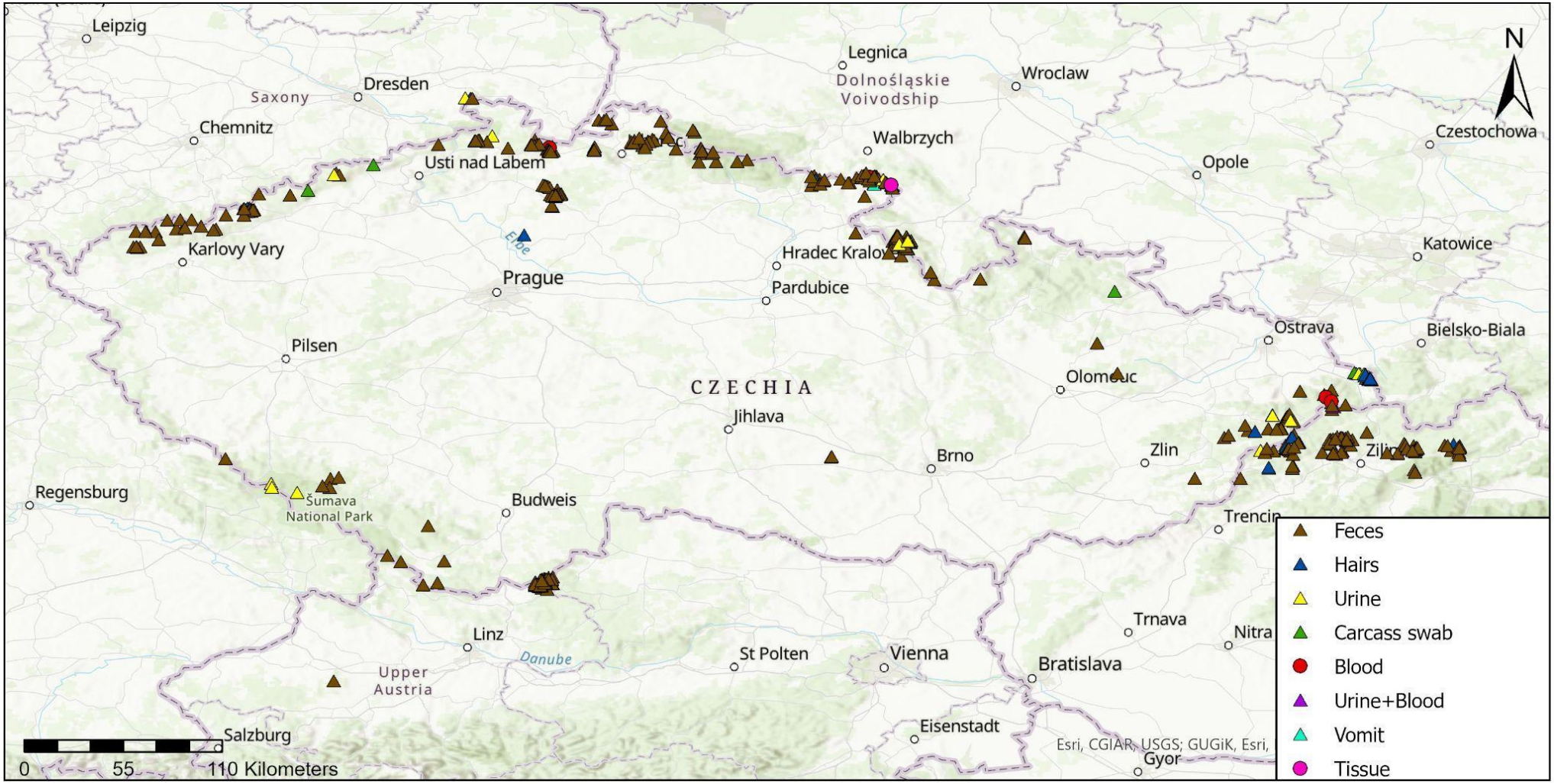

Figure 6: Locations of individual sampling collections with their sample types.

Samples were assigned into seasons based on the date of their collection. Wolf seasons start on the 1st of May and end on the 30th of April the following year. Wolf seasons or wolf years correspond to the wolf reproductive cycle, as most pups are usually born in April/May (Friends of the Earth Czech Republic 2022). Therefore, our study was able to fully cover 2 wolf years in 2020/2021 and 2021/2022 and partially season 2022/2023. (Fig. 7). Several samples belonging to the previous season (2019/2020) were also analyzed, however, their number was very low (only five samples) to represent any significant results.

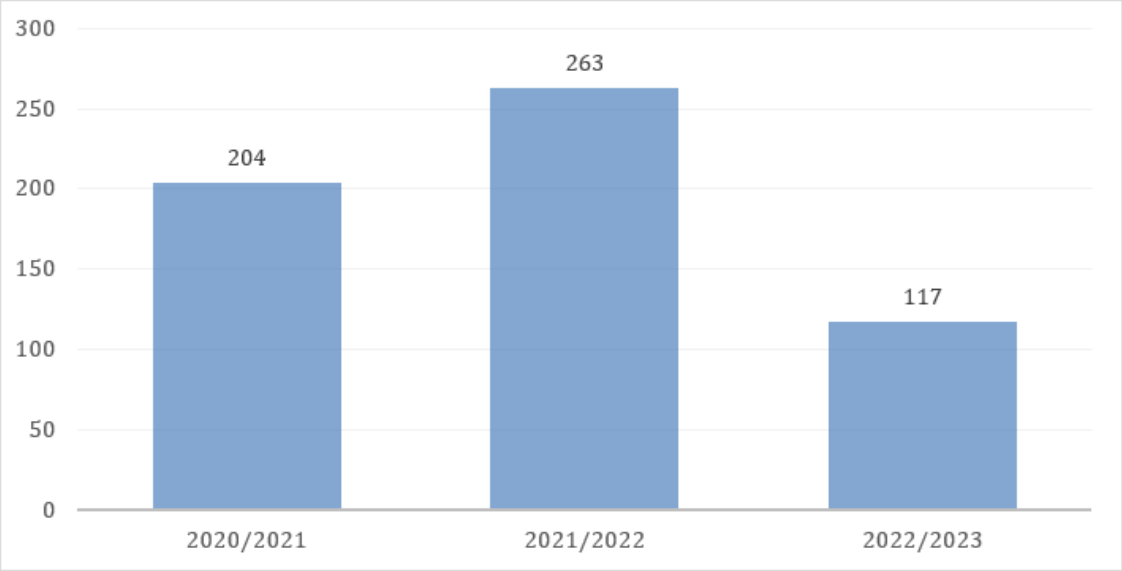

Figure 7: Number of samples collected during each wolf season (1.5. - 30.4.)

The majority of samples were gathered during the colder months spanning from October to April, as illustrated in Figure 8 which depicts the distribution of sample collection across different months.

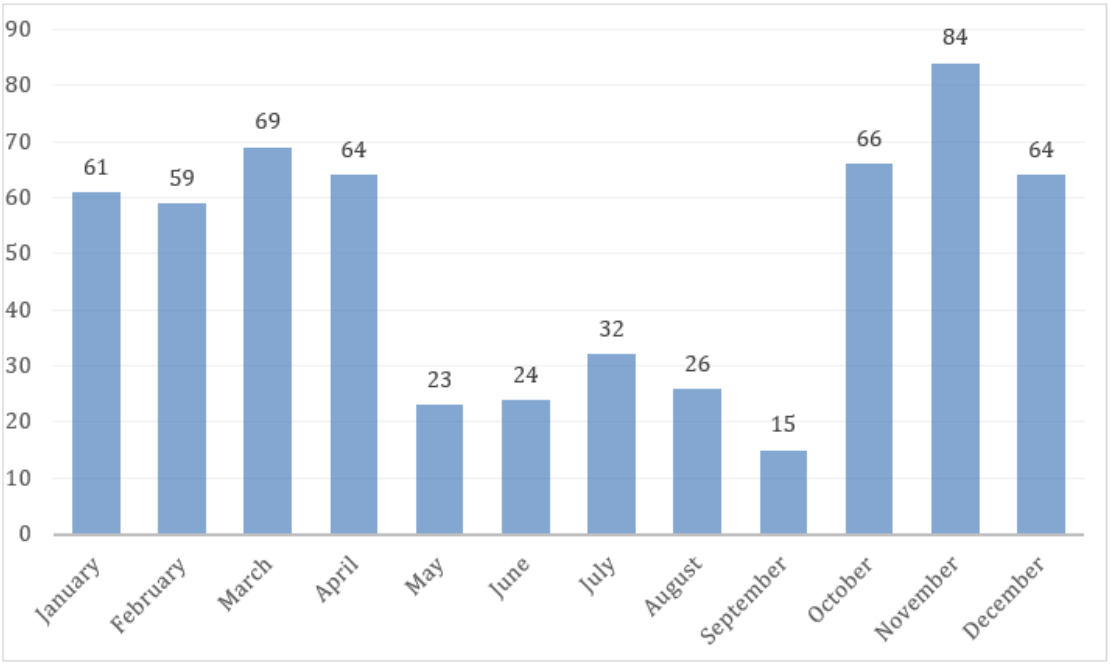

Figure 8: Number of samples collected in each month.

4.2. Genetic analyses

Analysis of 589 samples resulted in obtaining a total number of 413 genotypes with an amplification higher than 14 loci after using the multiple-tube approach.

The Bayesian Clustering analysis in software STRUCTURE 2.3.4. (Pritchard et al. 2000) distinguished 367 genotypes clustering with wolves, 29 genotypes of foxes, 15 domestic dogs, and 2 samples containing mixtures of genotypes (wolf/fox; Fig. 9).

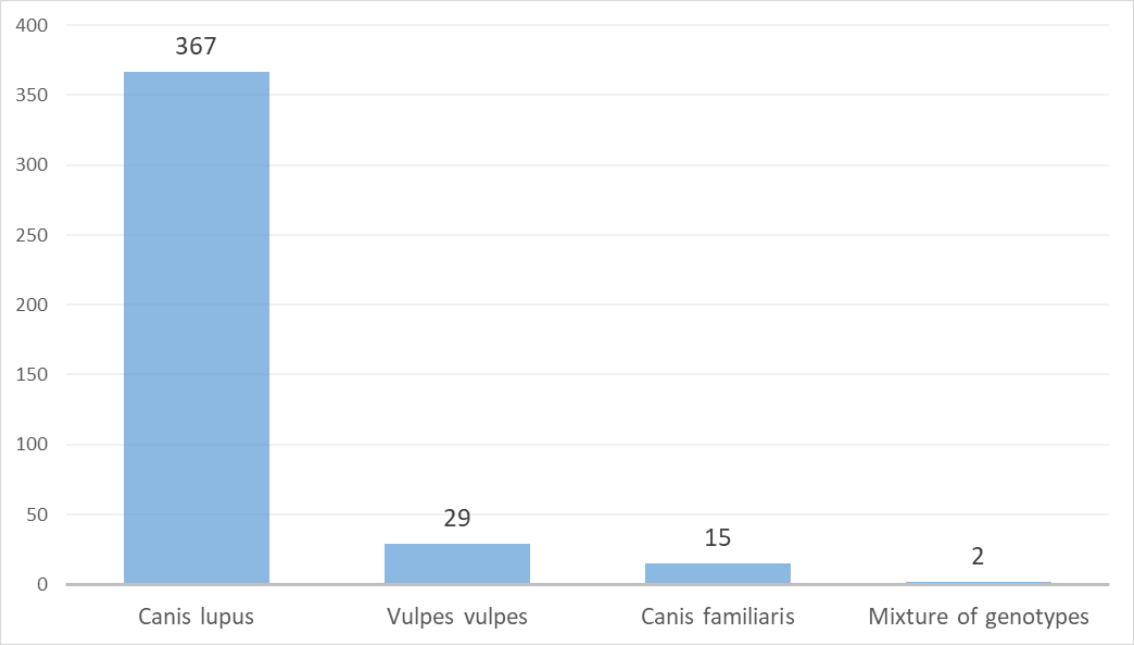

Figure 9: Species origin of tested genotypes.

Identity analysis revealed a total number of 183 unique wolf genotypes. The sex of the individuals was determined using the amplification of gene amelogenin with a ratio of 1.04:1 in favor of males (80 males, 77 females). In 26 genotypes (14%) it was not possible to determine the sex of the individual because amelogenin did not amplify.

Numerous recaptures were observed during the study, ranging from a single recapture to as many as 14 occurrences of a single individual (Tab. 3).

Table 3: Number of individuals with their number of recaptures throughout the entire study period.

Number of Individuals	Number of recaptures
38	1
20	2
9	3
3	4
7	5
0	6
0	7
0	8
0	9
1	10
0	11
0	12
0	13
1	14

In total, five (two females and three males) long-distance movements (>50km) were documented throughout the course of the study with individuals being recaptured as far as 250km from their initial sampling occasion (Fig. 10).

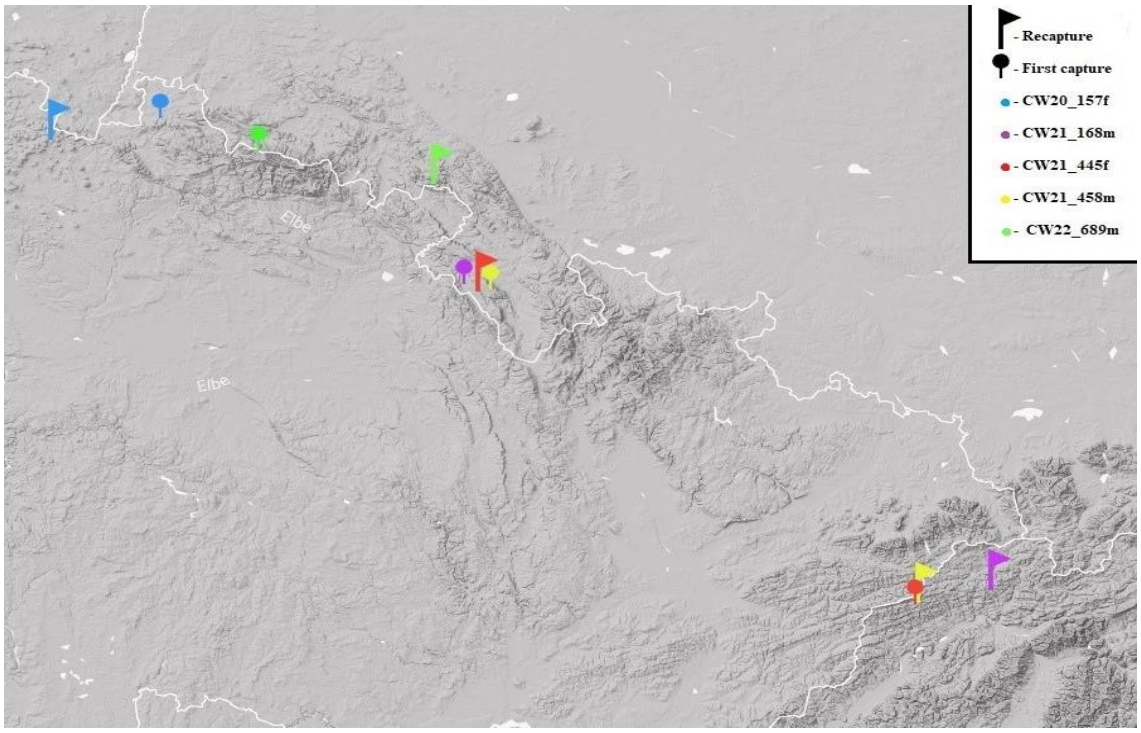

Figure 10: Map depicting five long-distance movements observed during this study.

The 183 unique genotypes were subjected to the Bayesian Clustering Analysis to determine their population origin. The best-supported number of clusters (populations) in the dataset was at K=4 (Fig. 11, 13) according to Li & Liu (2018). Those four clusters correspond to the presumed populations used in the dataset (Alpine, Carpathian, Central European) and the comparative dataset of dog genotypes. According to Evanno et al. (2005), the best-supported number of clusters was estimated at K=3 (Fig. 11, 13).

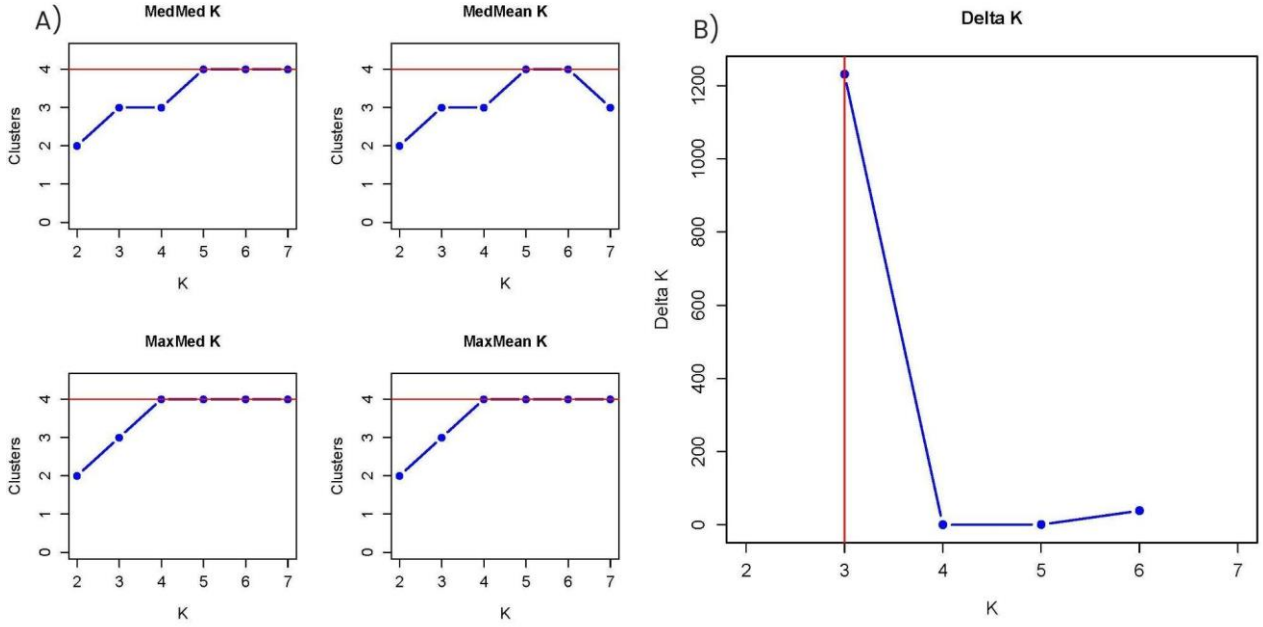

Figure 11: Results from STRUCTURE selector at 20 loci A) MedMed K and MedMean K. The red line suggests the best supported number of clusters at K=4 according to Li & Liu (2018). B) Delta K according to Evanno et al. (2005), suggesting the best supported number of clusters at K=3.

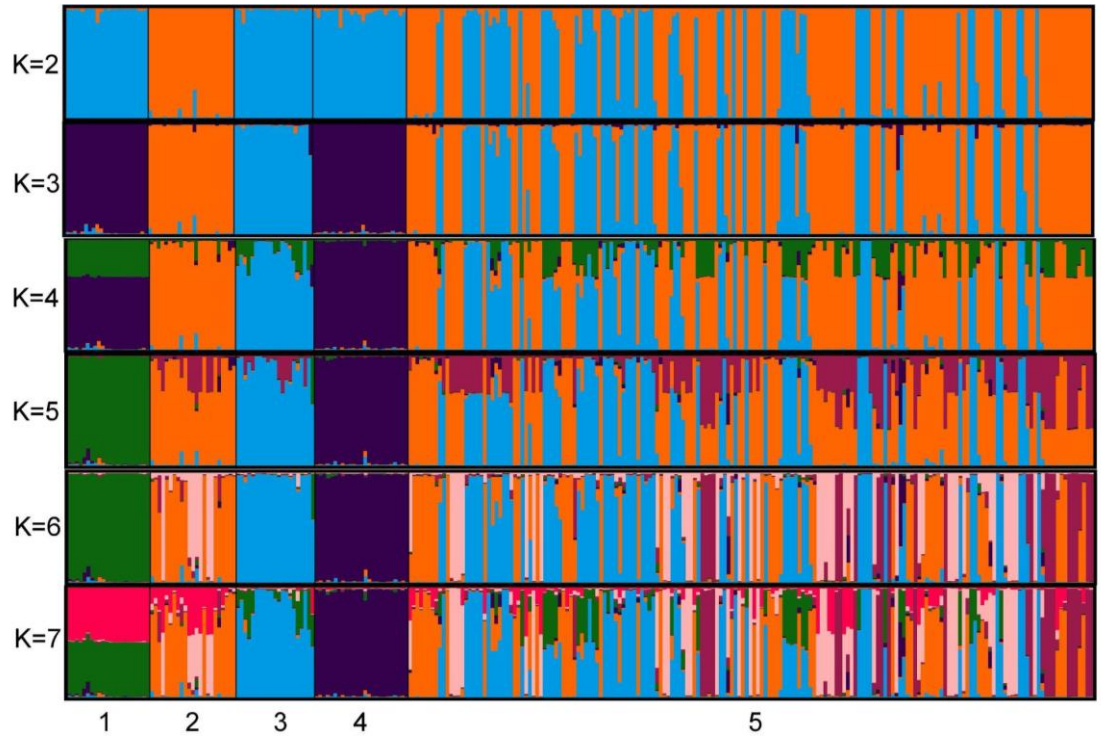

Figure 12: Results of population assignment using Bayesian Clustering analysis at 20 loci in STRUCTURE for K=2 to K=7. (1) Domestic dogs, (2) CE population, (3) Carpathian population, (4) Alpine population. (5) Tested genotypes.

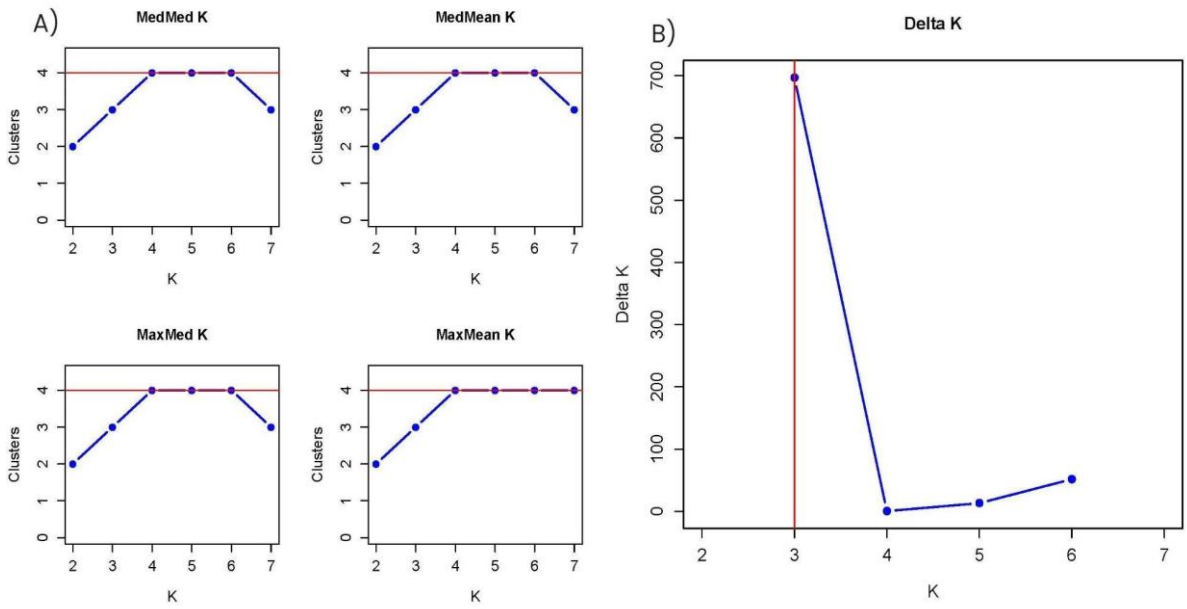

Figure 13: Results from STRUCTURE selector at 12 loci A) MedMed K and MedMean K. The red line suggests the best supported number of clusters at K=4 according to Li & Liu (2018). B) Delta K according to Evanno et al. (2005), suggesting the best supported number of clusters at K=3.

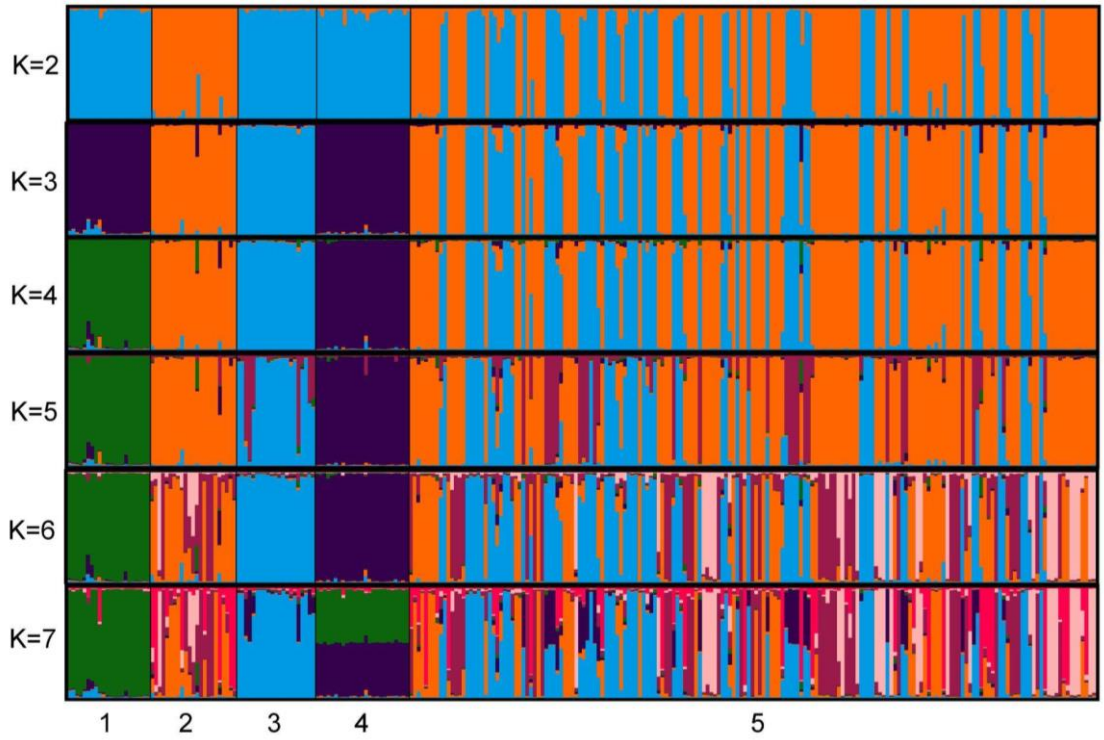

Figure 14: Results of population assignment using Bayesian Clustering analysis at 12 loci in STRUCTURE for K=2 to K=7. (1) Domestic dogs, (2) CE population, (3) Carpathian population, (4) Alpine population. (5) Tested genotypes.

After the initial analysis to exclude dogs and foxes, final analyses confirmed the absence of domestic dogs in our dataset and assigned the tested genotypes based on their membership coefficients to comparative populations (Fig. 12, 14). The majority of sampled individuals (115 individuals) were predominantly assigned to the Central European population (orange color). The smaller portion of genotypes from our dataset (68 individuals) predominantly clustered with the Carpathian population (blue color). Results also showed that none of the 183 tested genotypes appears to have an exclusive origin in the Alpine population. The distribution map of sampled individuals and their population affiliation based on the results of Bayesian clustering analysis can be seen in Figure 15.

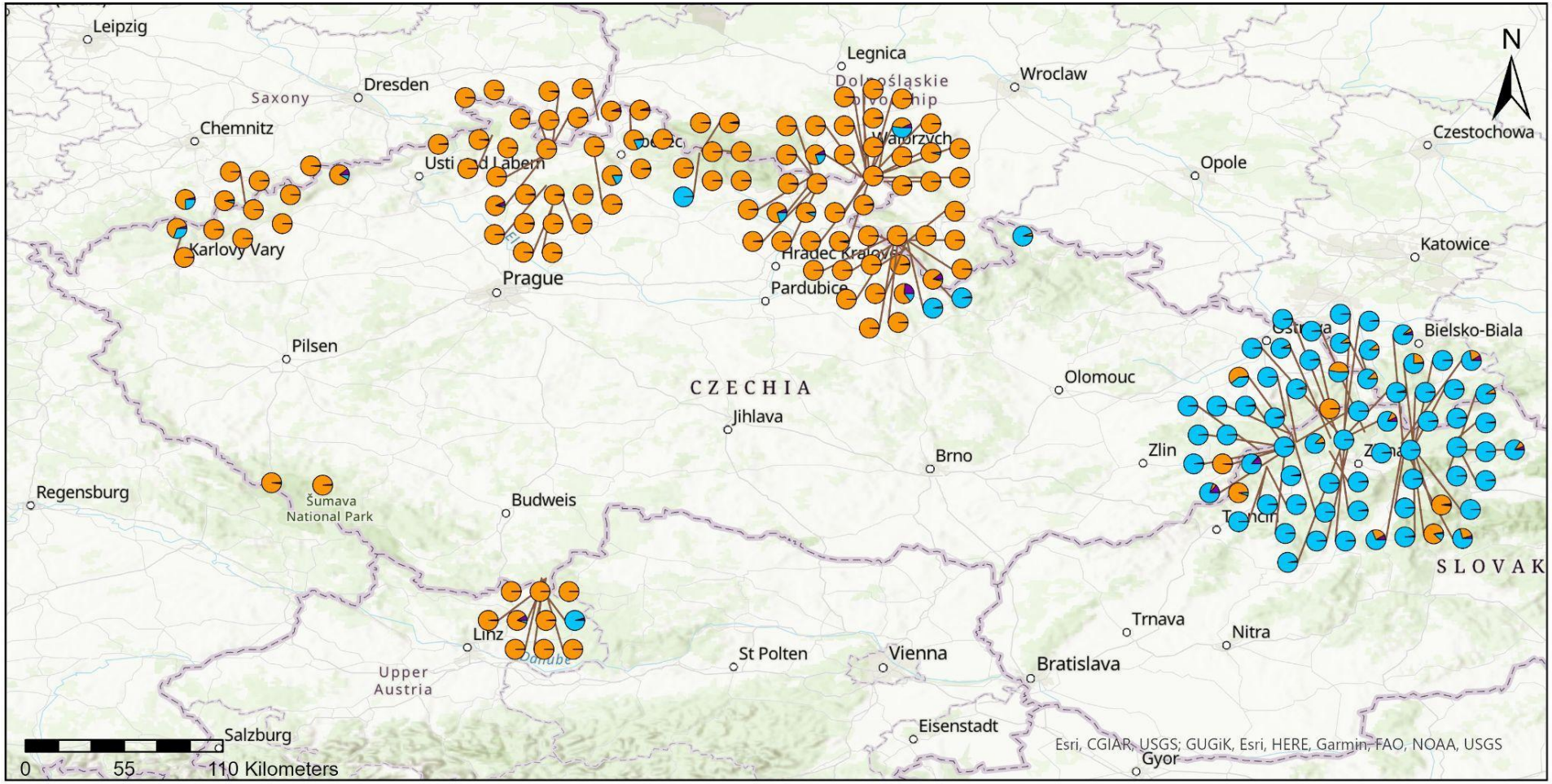

Figure 11: Results of Bayesian Clustering analysis at K=3 depicted on the map. Pie charts illustrate individual membership to the comparative populations. Color proportion within each pie chart corresponds to the relative membership coefficient obtained from the Structure analysis. Orange color represents the CE population, blue represents the Carpathian population and purple represents the Alpine population.

Results of PCoA demonstrated the dissimilarity of the Alpine population from the tested genotypes (Fig 16). The genetic variability among the populations is collectively explained by 25.99%, with individual contributions of 13.02% by the X axis, 7.04% by the Y axis, and 5.93% by the Z axis.

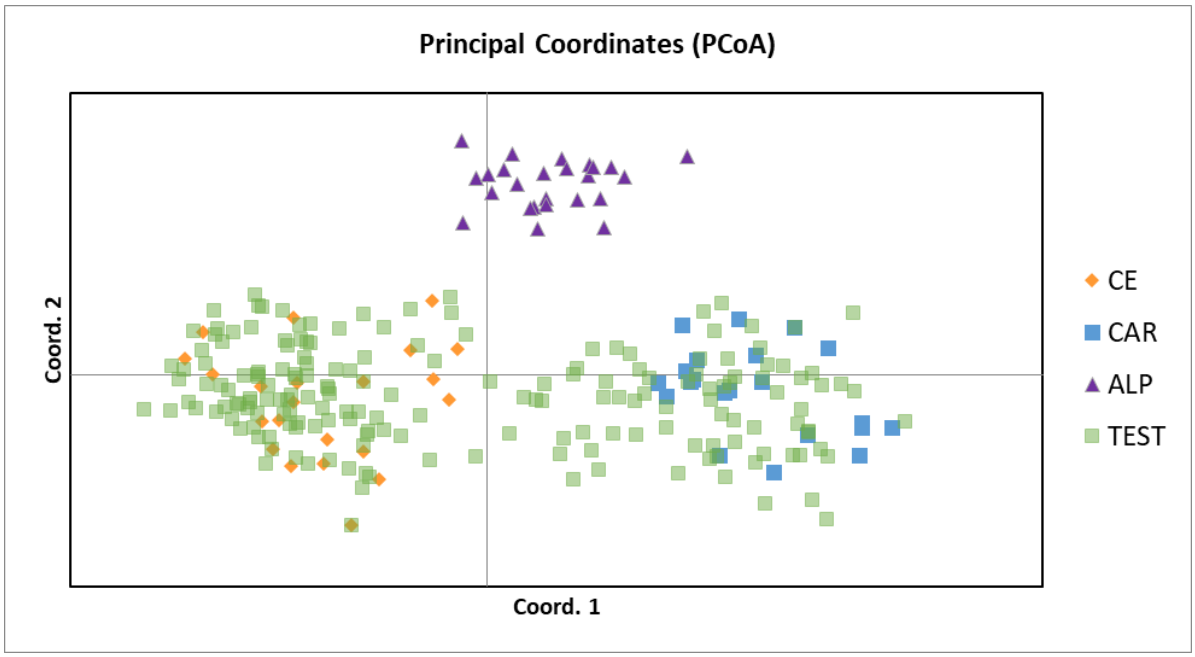

Figure 16: Principal Coordinates Analysis of three wolf populations and the tested samples.

Parameters of genetic diversity were calculated for each population present within the tested samples. Both populations exhibited lower observed heterozygosity than expected. The Carpathian population displayed higher values for the number of effective alleles, fixation index, and heterozygosity while maintaining a lower coefficient of inbreeding compared to the CE population. Both populations population exhibited statistical significance at a significance level of 0.000, indicating that they do not conform to the Hardy-Weinberg equilibrium (HWE), therefore the observed heterozygosity significantly deviated from the expected in both populations. The parameter values are presented in Table 4. No genotyping errors such as large allele drop out or stuttering were observed. However, several loci showed evidence for null alleles. In the CE population, null alleles were detected at seven loci (Locus 2, 5, 9, 10, 13, 16, 18), while in Carpathian

population, null alleles were present at three loci (Locus 1, 4, 17). Detailed information about the null alleles is provided in Appendix 1.

Table 4: Parameters of genetic diversity in observed populations. Ne=No. effective alleles, F=Fixation index, He=Expected heterozygosity, Ho=Observed heterozygosity, * statistical significance at p=0.000, indicating deviations from the Hardy-Weinberg equilibrium (HWE), F_{is}=Coefficient of Inbreeding

	Ne	F	He	Ho	F_{is}
Central European	3.107 ±0.252	0.090 ±0.022	0.621 ±0.042	0.554 ±0.035*	0.1078
Carpathian	3.441 ±0.283	0.097 ±0.022	0.655 ±0.037	0.594 ±0.037*	0.0931

.

The hybridization analysis in NewHybrids software (Anderson & Thompson 2002) did not discover any individuals with a hybrid origin within the tested genotypes. The results of the hybridization analysis are presented in Figure 17.

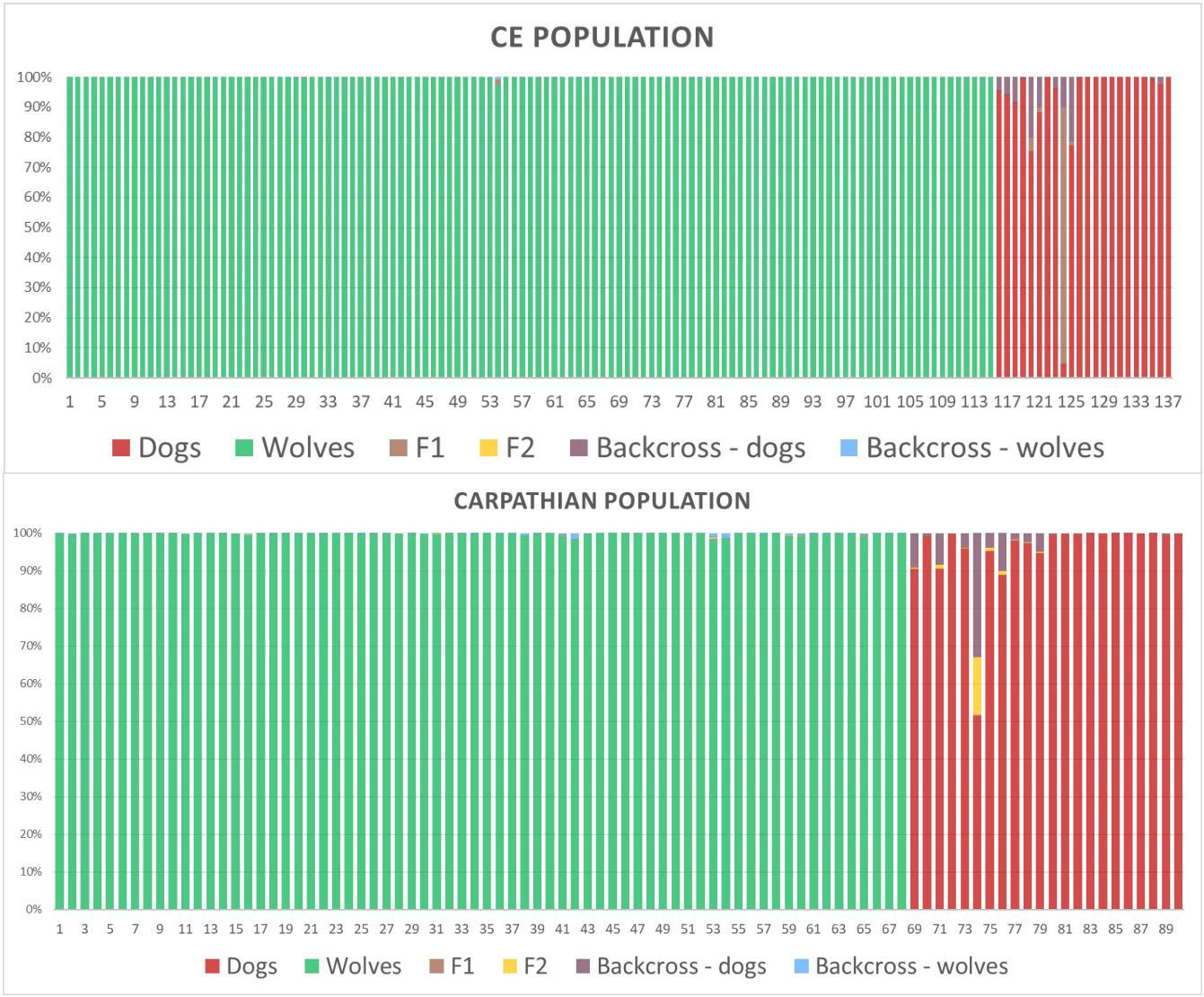

Figure 17: Results from NewHybrids software separately for the CE population and the Carpathian population. The graph depicts gene pool composition. CE population: 1–115 tested genotypes, 116–137 genotypes of domestic dogs. Carpathian population: 1–68 tested genotypes, 69–90 genotypes of domestic dogs.

5. Discussion

5.1. Monitoring efforts

Genotypes of wolves were successfully obtained from approximately 50% of the analyzed samples. This percentage aligns with the results from previous studies focused on the genetic monitoring of wolves such as 45% by Caniglia et al. (2012), 45% by Valentova (2021), 48.54% by Galaverni et al. (2012), and 53% by Dufresnes et al. (2019). However, it is important to note that each study employed a different number of microsatellites, and the criteria for considering genotyping successful based on the percentage of amplified loci varied as well.

Samples of blood and tissue exhibited high genotyping success, which was not surprising for these types of samples as they usually contain high-quality DNA (Ghatak et al. 2013; Ferreira et al. 2018). Among the remaining samples, the highest success rate was observed in samples of urine-blood mixture (100%), however, there were only five samples of this type. Samples of feces had a success rate of 50.25%, followed by hair samples (50%) and urine samples (40%). The lowest success rate was exhibited by carcass swabs, with a rate of only 11%. Nonetheless comparing success rates among different types of samples might be misleading due to the substantial disparity in the number of samples analyzed, ranging from 516 feces samples and only one tissue sample.

The lower success rate of carcass swabs may have been influenced by the low number of samples (only nine), as other studies such as Dufresnes et al. (2019) observed a significantly higher percentage (60%). However, it is possible that the results could have been affected by potential errors during collection such as improper technique, contamination, transportation issues, etc. This illustrates the challenges associated with collecting non-invasive samples and underscores the critical significance of employing proper techniques during the collection process as well as during all the following analyses.

This study comprehensively covered two wolf seasons spanning from May 1st to April 30th, in 2020/2021 and 2021/2022. However, due to logistical constraints, it was not possible to process all the samples collected during the third season in

2022/2023 and run all the necessary analyses before the submission of this thesis, thus, the last season was covered only partially.

From Figure 7, it is evident that most of the sampling was carried out during the colder month of the year (October till April). These months are generally considered more suitable as the environmental conditions in the Czech Republic are more favorable for sampling (slower DNA degradation, easier detection through snow tracking; Agetsuma-Yanagihara, Inoue, Agetsuma 2017), therefore wolf patrols are also more active in their sample collection during this period (Friends of the Earth 2023).

5.2. Species determination

When collecting samples in the field without direct observation of individuals, certain constraints must be addressed (Waits & Paetkau 2005) as it is almost impossible to distinguish noninvasive samples (scats, hairs) of wolves from those of other species. Although the primers employed in this study were originally designed for wolves, they have the capability to amplify genetic material from other closely related canids. Given the fact that red foxes and domestic dogs are commonly found in the same areas as wolves (Galaverni et al. 2012; Hoffmann & Sillero-Zubiri 2021), it was expected that some of the collected samples might belong to one of those species. The presence of two wolf/fox genotypes mixtures was most likely the result of interspecific contamination as canids are known to defecate over the feces of other species as part of their marking behavior (Mech & Boitani 2003).

5.3. Hybridization

Analysis of hybridization did not discover any individuals with a hybrid origin within the tested samples. Surprisingly, it did discover diversity in the gene pool composition among the comparative genotypes of domestic dogs, which was likely a result of the wide range of dog breeds present in the dataset. While no hybrid origin was found within the tested samples, the analysis required division of the samples based on their population affiliations (Fig. 14).

Although no signs of hybridization were detected in our samples, it is important to continue monitoring this issue, as it poses a threat to the genomic integrity of wolves (Hindrikson et al. 2017), especially in small and fragmented populations (Hindrikson et al. 2012). Despite this, individuals with hybrid origin have been reported in all nine extant wolf populations across Europe (Salvatori et al. 2020). Notably, there has been an increase in hybrid detection over the last two decades. However, this could be attributed to the increased implementation of genetic analyses. Nonetheless, several authors suggest that the increase in hybridization may have been driven by factors such as expansion into anthropogenic landscapes and locally high levels of human-induced mortality (Hindrikson et al. 2012; Galaverni et al. 2017; Donfrancesco et al. 2019) both of which enhancing the likelihood of encounters with domestic dogs.

5.4. Identity analysis

A total of 183 individuals were identified, many of those multiple times during the study period. However, many recaptures were the result of collecting multiple samples during one sampling occasion which later proved to be from a single individual. Additionally, the obtained data were unsuitable for any population size estimates due to the requirement of certain assumptions such as population closure and equal capture probabilities for all individuals, which our study did not fulfill.

The sex ratio (1.04:1) favoring males in our study corresponds to other studies. Although our observed ratio was lower compared to ratios such as 1.18:1 by Fabbri et al (2007), 1.33:1 by Veselovska (2023), and 1.36:1 by Marucco et al. (2009). The male bias in the sex ratio within our results aligns with the natural dispersal behavior of wolves, as males typically exhibit a greater tendency to disperse over larger distances (Stansbury et al. 2016; Marucco et al. 2022). Therefore, a male-biased sex ratio was anticipated, given the ongoing expansion from other areas. The predisposition of males was further supported by the discovery of long-distance movements in our study, as the majority of those were undertaken by males.

5.5. Population origin

Our results confirm the dominance of the CE population within the Czech Republic followed by the second most abundant Carpathian population which was revealed by previous studies (Dolejský 2021; AOPK 2023). Several factors are likely to contribute to the more rapid expansion of the CE population into the Czech Republic. The first factor is the high adaptability of the CE population, enabling it to thrive even in human-dominated landscapes with high population density (Hulva et al. 2018). Whereas wolves originating from the Carpathian region tend to exhibit a stronger association with forest-covered mountain areas (Find'o et al. 2008).

The smaller proportion of the Carpathian wolves in our results was also most likely influenced by distinct management approaches in Slovakia in recent years (Kutal et al. 2016), as wolves were subject to legal hunting in Slovakia until 2021 (MŽP SR 2021). Given the fact that wolves exhibit no consideration for human borders, hunting created a mortality sink not only for wolves in Slovakia but for wolves from other countries as well (Jedrzejewski et al. 2010). Killing one of the reproducing individuals frequently results in the disturbance of the entire pack's dynamics (Mech & Boitani 2003; Stansbury et al. 2016). Human-induced mortality has further been proven to reduce distance, duration, and overall success of dispersal due to the tendency of dispersing animals to avoid places where they might encounter humans, such as motorways, urban areas, and farmlands. Consequently, these human-impacted areas can pose additional dispersal obstacles, compounding the challenges posed by natural barriers (Morales-González et al.2022). Therefore, hunting practices in neighboring Slovakia emerged as a prominent factor impeding the expansion of Carpathian wolves into the Czech Republic and other countries (Selmy.cz 2023). Due to the transboundary nature of many wolf territories in the Czech Republic, the conservation status of wolves relies upon collaborative management efforts on both sides of the borders (Kutal et al. 2016).

On the contrary, wolves in Poland have been strictly protected since 1998, a factor that facilitated the rapid growth of the CE population and its expansion into other countries (Reinhardt et al. 2015; Kutal 2017; Schley et al. 2021, Boitani et al. 2022). Since wolves are now protected in Slovakia as well, it is possible to expect an

increase in the immigration of Carpathian wolves into the Czech Republic in the coming years.

In contrast to the findings of Hulva et al (2018), we did not identify any individuals with an exclusive origin from the Alpine population. Nevertheless, it was likely caused by the limited number of samples collected near the southern borders as this population is primarily expanding from the south (Hulva et al. 2018). The smaller sample size collected in the Šumava National Park, and the surrounding areas can be attributed to the already existing practice of monitoring large carnivores conducted by the national park personnel (Mokrý 2021). Hence, undertaking additional monitoring activities on our part would prove inefficient in terms of both time and resources, given the coverage provided by existing monitoring projects in this region. However, based on the currently available data, the number of wolves occupying the Šumava National Park is estimated to be approximately 36 individuals, distributed across 6 territories. These wolves predominantly belong to the Alpine and Central European populations, with sporadic occurrences of dispersing individuals originating from the Dinaric-Balkan population (NP Šumava 2023; AOPK 2023).

Based on our findings, we can anticipate individuals from the Carpathian population to be present in Bohemian Forest Mts. in the future as well, if not already. Given their presence in the Novohradské Mts., we can reasonably expect their further westward expansion. Additionally, it is important to point out that our study did not include comparative datasets of individuals from the Dinaric-Balkan population, therefore we recommend future studies to include individuals from this population to obtain more precise results.

Our results presented in Figure 16 distinctly illustrate the presence of individuals from different populations within the Czech Republic, indicating a certain level of gene flow and interactions across multiple locations (Novohradské Mts, Orlické Mts., Broumovsko, Krkonoše Mts. and possibly Krušné Mts.). This gene flow was further supported by the discovery of long-distance movements of individuals between Javorníky Mts. in Slovakia and Orlické Mts. in the Czech Republic (Fig. 9). These findings highlight dynamic interactions between populations and potential genetic exchange across Central Europe.

While the CE population exhibits a much stronger expansion trend compared to the Carpathian population, its genetic diversity is generally lower. Most likely as a consequence of a strong founder effect (Hindrikson et al. 2017; Hulva et al. 2018; Szewczyk et al. 2019) as it is presumed to have originated from a very small number of individuals (Czarnomska et al. 2013; Hindrikson et al. 2017). Our results correspond to this pattern, as the CE population exhibited lower values for the number of effective alleles, fixation index, and heterozygosity compared to the Carpathian population. Additionally, the CE population showed a higher value of the coefficient of inbreeding, a result of frequent mating between related individuals.

Both populations deviated from HWE, and several loci showed evidence for null alleles, nonetheless, each population exhibited null alleles at different loci (Appendix 1) and estimation of their frequency was lower than 0.13. Therefore, we assume that the presence of null alleles was not a consequence of genotyping errors, but rather a consequence of the departure from the HWE which could be caused by biological factors. Both populations were sampled at the edges of their distribution, and both are currently expanding. Factors like long distance migration, founder effect inbreeding, and genetic drift could have affected the population equilibrium.

6. Conclusions

With the increasing numbers of wolves across the continent, the importance of monitoring these animals is rising, particularly in human-dominated landscapes with a high probability of interactions with humans. This thesis was focused on the genetic monitoring of wolves in the Czech Republic and its neighboring countries as this region has experienced recent recolonization by wolves from multiple wolf populations. The objective was to assess information about the presence of wolves in the region and to determine the population origin of the detected individuals.

We observed that wolves are already present in most of the mountain ranges along the country's borders. In our dataset, we discovered individuals originating from two distinct populations. The majority of detected individuals clustered predominantly with the CE population, while the remaining individuals were assigned to the Carpathian population. Notably, individuals from the CE population exhibited lower genetic diversity, most likely a result of a strong founder effect. Our findings further demonstrated that the previously isolated populations have already encountered one another at multiple locations, indicating the presence of a certain level of gene flow between the populations.

Although our results did not detect any individuals predominantly originating in the Alpine population, other sources indicate that these individuals are present in the southern regions of the country. Our also study lacked the comparative dataset of individuals originating in the Dinaric-Balkan population. Therefore, we recommend that future studies incorporate this population to achieve more precise results, given the sporadic occurrences of individuals from this population within the country.

Furthermore, we recommend continuation of cooperation among the Central European countries in monitoring and conservation efforts of wolves and other large carnivores. Given the fact that these species do not respect political borders, such cooperation provides a better understanding of their movements and ecology and ultimately contributes to more effective conservation practices across the region.

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Appendices

Appendix 1: Table obtained from software Micro-Checker (Oosterhout et al. 2004) depicting information about the presence of null alleles within the tested genotypes.

CE population			Carpathian population		
Locus	**Null allele present**	**Oosterhout**	**Locus**	**Null allele present**	**Oosterhout**
Locus1	no	0,0367	**Locus1**	yes	0,0834
Locus2	yes	0,1187	**Locus2**	no	0,0624
Locus3	no	0,0274	**Locus3**	no	0,0034
Locus4	no	0,0103	**Locus4**	yes	0,1266
Locus5	yes	0,1075	**Locus5**	no	0,0164
Locus6	no	0,0047	**Locus6**	yes	0,0928
Locus7	no	-0,0088	**Locus7**	no	0,0698
Locus8	no	0,0328	**Locus8**	no	-0,0245
Locus9	yes	0,1034	**Locus9**	no	-0,0299
Locus10	yes	0,0941	**Locus10**	no	0,0704
Locus11	no	-0,011	**Locus11**	no	0,1297
Locus12	no	0,0175	**Locus12**	no	0,0219
Locus13	yes	0,078	**Locus13**	no	0,0558
Locus14	no	-0,0063	**Locus14**	no	-0,0922
Locus15	no	0,0419	**Locus15**	no	0,0516
Locus16	yes	0,0594	**Locus16**	no	0,0105
Locus17	no	-0,0068	**Locus17**	yes	0,0711
Locus18	yes	0,0987	**Locus18**	no	0,0474
Locus19	no	0,044	**Locus19**	no	0,0792
Locus20	no	0,0459	**Locus20**	no	0,0391