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Genetic structure of Grey wolves in Central Europe

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Declaration

I hereby declare that I have done this thesis entitled Genetic structure of Grey wolves in Central 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, 26.4.2019

.....

Alžběta Báčová



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Abstract

Central Europe is considered as a contact zone for many species, which underwent the allopatric differentiation in the past. Recently, we are witnessing natural recolonization of Czech Republic by wolves from neighbouring countries. Czech Republic lies in the centre of possible recolonization routes of distinct wolf populations. Czech population of wolves is rapidly increasing in size due to several factors such as legal protection, changes in landscape management or socio-economic factors. For sustainable management and protection, it is necessary to monitor the population. Genetic monitoring can answer the questions about the origin and relatedness of the wolves. We used 21 microsatellite loci and one sex-determining gene Amelogenin to reveal the genetic structure and relatedness of wolves in Central Europe, with special emphasis to northern Bohemia. The genetic analyses indicate dispersal pattern from Carpathians towards the protected area Broumovsko in the northeast of Bohemia. We found genetically distinct individuals in the South Bohemia, which were not clustered with Lowland or Carpathian populations. Haplotype distribution is consistent with previously published studies. The estimation of relatedness showed the several related individuals mainly in protected area Kokořín, where the first Bohemian wolf pack was established after one hundred years of their absence in the country.

Key words: *Canis lupus*, Central Europe, gene flow, genetic structure, population

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List of the abbreviations used in the thesis

LCIE – Large

MCMC – Markov Chain Monte Carlo

PA – Protected Area

PCR – Polymerase Chain Reaction

1. Introduction

1.1. Wolf ecology

The Grey wolf (*Canis lupus*) as highly mobile top predator inhabits wide range of ecologically diverse habitats. Historical occurrence of this species was across the Holarctic – from tundra to grasslands and deserts (Nowak, 2003). Wolves can disperse for several hundred kilometres (Mech & Boitani 2010; Van Camp & Glukie 1979; Fritts 1983; Mech 1987). It is the most widespread European large carnivore (Randi 2011).

Pilot et al. (2006) observed significant difference in gene pool among populations in Eastern Europe, based on geographic parameters, temperature and vegetation types. Both, mtDNA and microsatellite variability depends on latitude, but not on longitude. Temperature and vegetation types significantly correlated with genetic distance and 43 % of the genetic variation could be explained by these two factors over the influence of geographical distance.

Although, 20 % of genetic variability was explained by occurrence of red deer (*Cervus elaphus*) in wolf diet composition, the factor of prey availability was not significantly correlated with genetic distance (Pilot et al. 2006). On the other hand, Musiani et al. (2007) proved that North American wolves inhabiting tundra and taiga follow migratory pattern of their prey – barren-ground caribou (*Rangifer tarandus groenlandicus*), despite the territoriality of grey wolves in boreal coniferous forest. Generally, the occurrence of wolves within area is determined also by prey availability and the proportion of dominant prey species in wolf diet increases with species availability in the community (Jedrzejewski et al. 2012).

1.1.1. Landscape fragmentation

Because of the ability to disperse on large distances, wolves also cross many anthropogenic barriers, such as roads, highways, railways, urbanized areas and human settlements. It is known, that the habitat fragmentation influences wildlife populations

in demographic and genetic point of view (Trombulak & Frissell 2000; Froman & Alexander 1998).

Habitat fragmentation is one of the major threats to natural animal populations. Carnivores, such as a wolf, are the most responsive to this threat as they were widely persecuted (Crooks et al. 2011). The fragmentation causes limited gene flow that results in small isolated populations. No immigrant events within affected population increase the level of homozygosity and decrease the effective population size. Such populations are very sensitive to loss of genetic variability by genetic drift. Highly homozygous individuals might not be able to respond to environmental changes, which, finally, can lead to extinction of entire population. Low densities of individuals force the animals to mate with their relatives, which can result in inbreeding depression that decreases the fitness of individuals as a consequence of accumulation of detrimental alleles.

Despite of previous studies, Gula et al. (2009) and Theuerkauf et al. (2007) provided evidence, that wolves in the Bieszczady Mountains, Poland are able to manage anthropogenic disturbances and are habituated to human presence. Although, habitat (forest) fragmentation is reported as one of the main factors of wolves' decline next to poaching (Mech & Boitani 2010), they are able to move through high human and road densities, and human settlements. Another example of wolves' adaptability to the local conditions is their recolonization of Czech Republic by individuals from Poland, Germany and Slovakia. Lowland wolves habituated highly fragmented Central Bohemian Region which is densely inhabited by humans (Hulva et al. 2018). After all, Geffend et al. (2004) use wolf as a model species for testing the prediction that population genetic structure is independent of landscape features. The wolf is able to respond to changing environmental conditions and anthropogenic barriers.

1.2. Phylogeography of wolves

The wolf, historically widely distributed across northern hemisphere, shows some rare patterns in haplotype structure. These patterns can be explained by historical processes in late Pleistocene, at the end of last glacial. Leonard et al. (2007)

compared ancient DNA of eastern Beringian wolves to modern North American wolves. There was not found any common haplotype. On the other hand, these ancient eastern Beringian wolves shared a common haplotype with some ancient European wolves (Pilot et al. 2010). According to morphological data of Leonard et al. (2007), ancient Beringian wolves preyed mainly on megafaunal species *Equus lambei* and *Bison bison*. The extinction of these two species at the end of last glacial led to extinction of specialized ecomorph of Beringian wolves. This observation denotes that the Pleistocene panmictic wolf population in Northern Eurasia and America ecologically and genetically varied from extant wolves in this area.

Pilot et al. (2010) defined two main haplogroups, 1 and 2, that represent a major subdivision of wolf population worldwide. According to previously mentioned author, the extinct eastern Beringian wolves belonged to the haplogroup 2, which is completely extinct in North America, nowadays. After this North American phenomenon, the same haplogroup substantially decreased in frequency in Europe at the same time.

Nowadays, Eastern Europe is predominated by haplogroup 1 in 87 % of individuals and the wolf populations have more than one haplotype (Pilot et al. 2006). On the other hand, Iberian Peninsula in Western Europe is strongly dominated by haplogroup 1. According to the latitude, the frequency of haplogroups does not show any pattern, but in Southern Europe (Balkan, Apennine and Iberian Peninsula) shows more unique haplotypes in both haplogroups (Pilot et al. 2010).

1.3. European distribution of wolves

Wolves' history in Europe is very rich and dynamic. In 18th and 19th century, this species was greatly persecuted and eradicated from most of the parts of Western Europe. Several individuals survived in fragmented populations in Iberia and Italy (Breitenmoser 1998). Also, the situation struck the Scandinavia population, where the wolves went extinct in 1960s (Vilà et al. 2003).

The natural re-expansion started slowly in the second half of 20th century, due to innovative law, socio – economic changes and the recovery of wild ungulates (Randi 2011). However, population in the Alentejo region in Southern Portugal (Álvares 2004) and small isolated population in Sierra Morena in Southern Spain (LCIE 2019) went extinct. Current wolf dispersion in Europe is displayed in Figure 1.

Scandinavian population underwent huge decline between 19th century and 1960. Despite of legal protection since 1966 in Sweden and since 1973 in Norway, the population was considered as functionally extinct at that time (Wabakken et al. 2001). The population was re-established in 1980s by just one immigrant pair from Karelia, Finland (Vilà et al. 2003). The population was highly inbred due to isolation by geographical distance (Wabakken et al. 2001, Vilà et al. 2003). It has been suffering from continuous inbreeding until 2007, when two immigrants from Finland genetically rescued this population. Repeated mating led to decrease of inbreeding level and to rapid increase of number of individuals (Åkesson et al. 2016). Nowadays, this population counts approximately 430 individuals (LCIE 2019).

Karelian population is established at the borders of Finland and Russia – Karelia. It is divided into Finnish and Russian subpopulation as the genetic analyses observed a low diversification (Aspi et al. 2006). Authors of the study also suggest possible initiation of population substructuring, because of different prey-preferences. Wolves in the southern parts prey on moose (*Alces alces*), meanwhile others prey on reindeers (*Rangifer tarandus*). Estimated number of individuals in 2016 was 200 (LCIE 2019).

Baltic population is distributed throughout Estonia, Latvia, Lithuania and north-eastern Poland. Despite of recent bottleneck in Estonia, Latvia and Russia (Hindrikson et al. 2013; Plumer et al. 2016; Sastre et al. 2011), this population show relatively high levels of heterozygosity in comparison to many others European populations (Jedrzejewski et al. 2005; Baltrūnaitė et al. 2013; Czarnomska et al. 2013; Hindrikson et al. 2013). This population serves as a valuable source for the Lowland populations as it reaches northern-eastern Poland (Czarnomska et al. 2013). The population size is estimated to 2240 individuals (LCIE 2019).

Dinaric-Balkan population is present in eight countries: Albania, Bulgaria, Bosnia and Herzegovina, Croatia, Greece, Serbia, Slovenia and Macedonia (Chapron et al. 2014). This situation allows to compare diverse array of monitoring and management approaches (Kaczensky et al. 2013). The population serves as valuable source of genetic diversity for neighbouring populations, as deduced by ongoing recolonization of the eastern and central Alps (Fabbri et al. 2014, Ražen et al. 2016). Bakan et al. (2014) also described gene flow between Balkan and Carpathian populations. The population might recover from highly fragmented structure by immigration and better protection (Bakan et al. 2014), but the sub-structuring is still present (Fabbri et al. 2014). Current number of individuals is 4000 (LCIE 2019).

Also, **Iberian population** did not avoid to several bottlenecks during the first half of 20th century and the population disappeared from most of its former range (Álvares 2004). This phenomenon resulted in observed low effective population size and increased risk of inbreeding (Sastre et al. 2011). In 2007, population counted 2500 individuals (LCIE 2019).

Apennine population, situated along the Apennine Mountains, consists of three genetic subpopulations (northern Apennines, central Apennines and southern Apennines). The gene flow within peninsula is quite limited (Scandura et al. 2011). This population arose from less than 100 individuals (Zimen & Boitani 1975). Italian wolves genetically differ from all other wolf populations worldwide (von Holdt et al. 2011) and carry two unique mtDNA haplotypes (Randi et al. 2000; Montana et al. 2017). Such a differentiation led to description of a new subspecies *Canis lupus italicus* (Altobello 1921). Also, Stronen et al. (2013) observed north-south differentiation within European wolves, where those Italian ones significantly differ from the rest of sampled population. It is assumed that Italian wolf population was isolated for thousands of generations (Pilot et al. 2010). Such an isolation supports the hybridization with feral dogs. Randi (2008) detected approximately 4-7% hybridization between wolf and dog. Nowadays, the population counts 2400 individuals (LCIE 2019).

1.3.1. Central European populations

Central Europe is unique in its position. This area is a crossroad for many populations of different species. Such a pattern was reported in hedgehogs (Bolfíková & Hulva 2011), house mouse (Selander et al. 1969), roe deers (Olano-Marin et al. 2014) and many other organisms including wolves (Pilot et al. 2010). This phenomenon is a result of climate oscillation during the Ice Ages. The climate changes along with the anthropogenic fragmentation caused the change of spatial distribution of species, which started to evolve allopatrically. This resulted in creating genetically different ecomorphs (Hewitt 2004).

Alpine population consists of wolves from four countries – Austria, Switzerland, France and Italy. The recolonization started from two sources. The Italian wolves recolonized western parts (Italy in 2000, Switzerland in 1996 and France in 1992) (Lucchini et al. 2002; Valière et al. 2003; Fabbri et al. 2007, 2014) probably through narrow corridor with suitable habitat of the Ligurian Apennines (Fabbri et al. 2007). The eastern and the central Alps were inhabited by Italian and also Dinaric-Balkan wolves (Fabbri et al. 2014, Ražen et al. 2016). According to Fabbri et al. (2007), the population was established by 2 to 20 unrelated individuals, respectively 8 – 16.

Since then, Alpine wolves reflect low genetic diversity which is caused by moderate bottleneck followed by founder effect. Despite potential high dispersal, all mentioned before is consequence of limited gene flow between Alpine part and rest of Italy (Fabbri et al. 2007).

Fabbri et al. (2014) revealed the origin of wolves in eastern Alps (Austria). The Apennine and Balkan-Dinaric (especially Croatian) wolves independently inhabited Austrian Alps. This phenomenon is clearly observed in Hulva et al. (2018) where Austria is occupied by both haplogroups from different populations.

Carpathian population is unique in its size and is considered as a potential link between northern and southern populations. Also, this population play key-role for the long-term survival of the species in Europe as the Carpathian Mountains constitute one

of the largest wolf refuge areas in Europe (Gula et al. 2009). The population is continuous with small fragmentation at the peripheral areas (Hindrikson et al. 2016).

Generally, Eastern Europe (including Carpathian population (Hulva et al. 2018)) is dominated by haplogroup 2. Haplogroup 1 was observed in lower frequencies (Pilot et al. 2010). Hulva et al. (2018) detected haplotypes W1, W2 and closely related W6 and W14. W6 and W14 dispose by the overlapping distribution. In Western Carpathians, haplogroup 1 was identified in small and isolated areas. Western part of this population is characteristic by low mitochondrial DNA variability as a consequence of recent bottleneck, common in many parts of Europe, and limited dispersion (Hulva et al. 2018). Both, author of previous study and Pilot et al. (2006) agreed on specific environmental conditions that genetically differentiate wolves within studied area.

This population is positively affected by legal protection in Poland and Czech Republic. In Slovakia, Romania and Bulgaria, wolf is considered as game species with strict hunting season. In Ukraine, wolf is hunted all over the year without any regulation (Salvatori & Linnell 2005). The population size is estimated up to 4000 individuals (LCIE, 2019).

Central European lowland population was settled in late 1990s in area of Germany-Poland borders (Andersen et al. 2015) by wolves from north-eastern Poland (Czarnomska et al. 2013). Since 2001, the population in western Poland has been increasing every year (Nowak 2016). Recolonization of Saxon area, Germany started in 2000 with 36% yearly increase. As stepping-stones, wolves primarily inhabited former military training areas and their surroundings rather than protected areas (Reinhardt et al. 2019). In November 2012, dead canid-like individual was found in Jutland, Denmark. After genetic analysis by Andersen et al. (2015), the wolf ancestry and its origin in Saxon, Germany was confirmed. This study proved long-distance dispersion such as Slovak wolf killed on highway in Czech Republic (Hulva et al. 2018).

This population is dominated by haplogroup 1 (Pilot et al. 2010) and carry W1, W2, W3, W6, W8 and W14 haplotypes (Czarnomska et al. 2013). Haplotypes W1, W2, W3 and W8 belong to haplogroup 1 common for north-eastern and central Europe and

the Iberian Peninsula. W6 and W14 are linked to haplogroup 2 that dominates in south-eastern Europe and Italy (Pilot et al. 2010).

The area of Poland is typical for its 'wolf-free belt' which separates Lowland and Carpathian populations (Czarnomska et al. 2013). This isolation of populations developed in distinct wolves' ecotypes. In Lowlands, wolves have smaller skull and females are bigger in comparison to Carpathian population. Generally, sexual dimorphism was found in both populations (Okarma & Buchalczyk 1993). These two populations also differ in habitat preferences and prey species (Pilot et al. 2006; Czarnomska et al. 2013).



Figure 1: Wolf dispersion in Europe (Hindrikson et al. 2016).

1.3.2. Wolves in Czech Republic

1.3.2.1. History of Czech wolves

As previously mentioned, European wolves underwent huge population decline resulting in several bottlenecks due to hunting and persecution.

In Czech Republic, wolves were abundant until mid17th century. Since then, the population had decline. In Bohemia, the last wolf was shot in 1874 in Šumava (Anděra & Červený 2009). As the last wolf in Moravia and Silesia is considered an individual shot in 1914 in Beskydy Mountains (Andreska & Andresková 1993; Anděra & Červený 2009). The data can diverge according to different sources of information, evidences and reliability. After the last shots, wolf was observed sporadically at Czech borders usually with poor evidences (Anděra & Červený 2009).

1.3.2.2. Current situation of Czech wolves

In the second half of 1990s, an evidences proved wolf presence in Beskydy Mountains (Bartošová 1998), later also in Šumava National Park (Bufka et al. 2005). Still, the presence was sporadic.

The first evidence of wolf permanent presence were camera trap pictures of young wolves taken in August 2014 in PA Kokořín (Friends of the Earth 2014). This evidence confirmed presence of pair and even their mating. Since then, wolves inhabited several areas within Czech Republic. This phenomenon is well-documented by thorough monitoring. In 2015, the first wolf was camera trapped in PA Broumovsko. Mating was confirmed later and since then, every year (Friends of the Earth 2018e). This pack was established by wolves from western Poland (Friends of the Earth 2015). In 2016, camera took a picture of individual in Jeseníky Mountains and near Šternberk town in 2017 (Friends of the Earth 2017a, 2017b). In December 2017, wolf was photographed in Bohemian-Moravian Highlands (Friends of the Earth 2017c). The pictures from Jeseníky Mountains and Bohemian-Moravian Highlands are the only proofs. There are no genetic evidences of wolf presence in this area. Probably, they

were vagrant individuals. In 2018, wolves inhabited several areas. From PA Třeboňsko came evidences of established pack (Friends of the Earth 2018a). One pair was observed in Czech-Switzerland National Park (Friends of the Earth 2018c). Beskydy Mountains and Krušné Mountains confirmed wolf packs too (Friends of the Earth 2018b, 2017d). Migratory individual was camera trapped in Lužické Mountains (Friends of the Earth 2018c).

Czech Republic is unique in its location in the centre of Europe. At the same time, populations could possibly meet here. Study revealed the Lowland origin of wolves in northern parts of Czech Republic, meanwhile Moravia and Silesia is inhabited by wolves from Carpathian Mountains (Hulva et al. 2018).

Wolves which are coming to Czech Republic show long-distance dispersion prepositions. Female hit by a car at D1 highway near Jihlava came from Carpathian Mountains (Hulva et al. 2018) and male killed by car on highway near Mladá Boleslav came from Poland (Friends of the Earth 2018d).

Nowadays, Friends of the Earth (2019) confirmed 16 wolf territories mainly at Czech borders (Figure 2).

Wolves in Czech Republic 2018

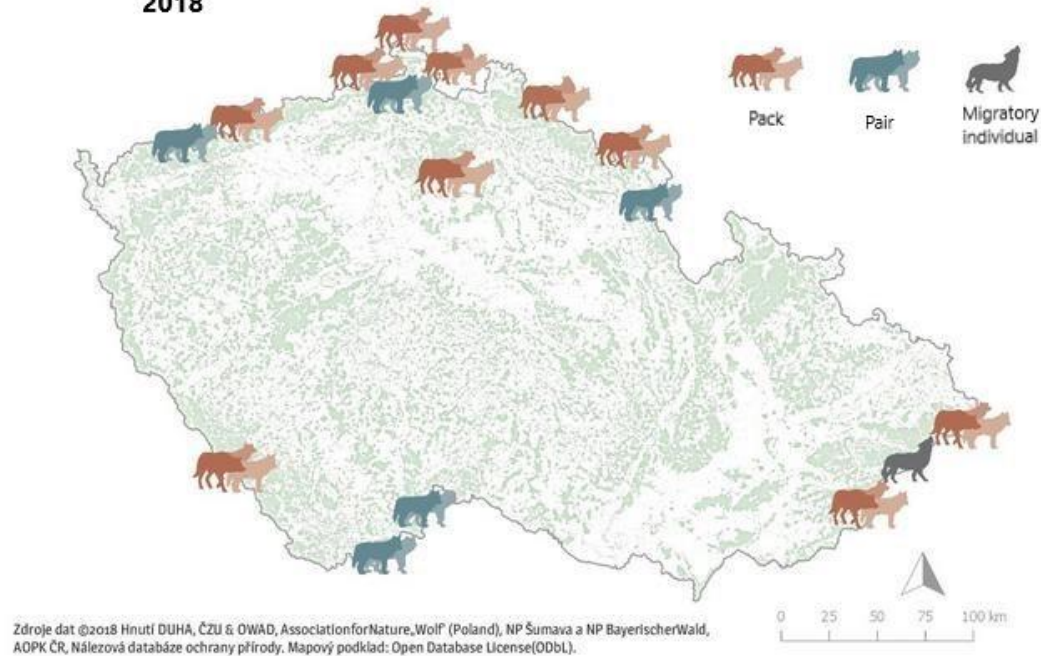


Figure 2: Wolf distribution in Czech Republic, 2018 (Friends of the Earth, 2019).

1.4. Genetic monitoring

As wolves are returning back to Czech Republic and many other countries, the need of monitoring is increasing. It is important for the right management and adequate legal protection, because wolf can be easily reported as a dog and *vice versa*. The most appropriate way how to identify the species is by genetic analysis and monitoring.

Genetic monitoring is highly used tool in management and conservation of populations especially of endangered and elusive species (Piggott & Taylor 2003). Meanwhile populations are complex in structure, they can change in time (reviewed in Milligan et al. 2018). Populations can expand, inhabit new geographical ranges, decline etc. Revealing population structure helps to make decisions that would be difficult to observe otherwise (*i. e.* Ovenden et al. 2015; Leblois et al. 2014). The assumption of genetic structure can be very important for every species that face to the risk of

extinction. Also, it reveals ecology of studied species, relationships and place of origin or even cryptic species, when the specimens do not differ phenotypically (Bastos et al. 2011).

The genetic monitoring provide us with unique data about the origin, history and relationship of individuals (Waist & Paetkau 2005). It is possible to continuously monitor and measure the genetic parameters, such as level of inbreeding or other population genetic parameters that refers and inform the conservationists about the success of their management (DeMay et al. 2017).

1.4.1. Non-invasive genetic methods

Non-invasive genetic methods of sampling are very popular in wildlife biology, despite the lower yields in comparison to invasive methods. Since the introduction of these methods in 1992 (Höss et al. 1992, Taberlet & Bouvet 1992), species-specific approaches were evolved. Although non-invasive genetic sampling methods were greatly accepted by wildlife biologists, genetic researchers pointed out several weaknesses and biases – increased fragmentation and lower quality of DNA can lead to high genotyping error rates such as allelic drop-out or amplification of false alleles (reviewed in Waits & Paetkau 2005, Pereira et al. 2009). To avoid these biases, it is necessary to repeat the PCR reaction (Pereira et al. 2009). These methods allow genetic studies of free-ranging animals without any need to capture or even see them and are very useful in the monitoring of rare and elusive species as for example snow leopards (Janečka et al. 2008) and Pyrenean desman (Gillet et al. 2016). Usually, faeces, urine or hairs are used (f. e. Huber et al. 2003; Sloane et al. 2000; Valière & Taberlet 2000). It is possible to extract the nuclear and mitochondrial data. From them, it is possible to gain the information about the genotype of the animal, the gender and it is also possible to identify the species. Non-invasive genetic methods are very popular across the studies regarding the wolves as a long-distance dispersal and elusive species (*i.e.* Santini et al. 2007, Stenglein et al. 2010, Dufresnes et al. 2019).

2. Aims of the Thesis

Summary of current situation of wolves in Central Europe and its assessment. The aim of practical part is to describe genetic structure and variability within wolves in Bohemian area. Also relatedness of observed individuals and their origin. Furthermore the comparison between distinct localities within this area.

3. Methods

3.1. Sampling

The non-invasive samples were collected by volunteers from Friends of the Earth during the years 2017, 2018 and the beginning of 2019. The volunteers are trained to identify and distinguish signs of wolf' presence as tracks or faeces. The area of interest covers entire Czech Republic, mainly mountains at the borders. Many locations are investigated using camera traps. In total, 169 samples was obtained, but one of them was excluded for low sample quality. Finally, I analysed 166 faeces samples, one buccal swab sample and one tissue sample. Buccal swabs and tissue were collected from dead individuals, mainly hit by cars. The list of isolated samples is in Appendix 1. The samples are stored in 96 % ethanol in freezers.

As comparable data sets were used Slovakian and Polish wolves, published in Hulva et al. (2018). Dogs were represented by 9 individuals from Poland (published in Hulva et al. 2018) and 30 pure-breed dogs sampled in recent years – 8 Czechoslovakian Wolfdogs, 8 Golden Retrievers, 5 Hollandse Herdershonds and 9 Labrador Retrievers.

3.2. Used markers

Autosomal nuclear microsatellites, widely used biparental genetic markers (Avice 2004), are short tandem repeats (STR) of non-coding DNA, firstly used in early 1990s (Ellegren 1991). The motifs of size 1 – 6 bp form blocks of up to 100 bp (Strachan & Read 1999). Huge advantage of these markers is their neutrality to natural selection and high mutation rate. Large number of loci enables more precisely estimate the level of polymorphism (Roy et al. 1994). Microsatellites can be used to analyse genetic diversity and gene flow, to distinguish individuals from one another, to determine relatedness among individuals and to reveal the population structure. These markers are more suitable for studies focused on recent past. In wolf population studies, these markers are widely used because of large numbers of characterised loci in the

3.3. Laboratory processing

DNA from faeces was extracted according to protocol of QIAamp® DNA Stool Mini Kit produced by QIAGEN. This extraction kit is based on pH changes at the silica gel membrane. The membrane releases the molecules of DNA in the last step by using the elution buffer or water. We used 100 µl of elution buffer to elute genomic DNA. DNA from buccal swabs was extracted according to protocol attached in Presto™ Buccal Swab gDNA Extraction Kit produced by Geneaid. In the last step, genomic DNA was eluted to 100 µl of elution buffer. The tissue was processed with DNeasy® Blood & Tissue Kit by QIAGEN. In the last step, genomic DNA was eluted to 100 µl of elution buffer.

To amplify specific microsatellite loci, it is necessary to run PCR (Polymerase Chain Reaction) with specific primers. We used 21 fluorescently labelled primers (FH2088, FH2054, FH2087, PEZ17, FH2017, FH2001, INRA21, REN169D01, FH2097, CXX279, REN169O18, FH2096, FH2137, INU055, VWF, FH2161, AHTk211, CPH5, FH2010, REN64E19, FH2140) and one sex-determining gene Amelogenin, that is located on gonosomes. The primers were divided into two primer mixes according to their fluorescent label and length range. The exact multiplex composition and parameters of primers are given in Table 1 and Table 2. The PCR contained 5 µl of PCR Mastermix (QIAGEN), 3 µl of RNA-free water, 1 µl of primer mix and 1 µl of genomic DNA. The PCR was run on Bio-Rad thermal cycler T100. The conditions of thermal cycle were as following:

1. 95°C, 5:00
2. 95°C, 0:30
3. 60°C, 1:30
4. 72°C, 0:30
5. GO TO step 2, 28x
6. 60°C, 30:00
7. 12°C, ∞

Fragmentation analysis was run at the service laboratory at Faculty of Science of Charles University on a sequencer ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems) with polymer POP4 and standard DS-33. Total volume of analysed mix was 10 µl – 8.5 µl of formamid, 0.5 µl ladder Gene Scan™ 500 LIZ Size Standard (Applied Biosystems) and 1 µl of PCR product. The PCR reaction was repeated again for samples that were amplified successfully to prevent occurrence of false homozygotes. Number of repeats was dependent on the matches and mismatches of the genotype.

Samples, which were successfully amplified on microsatellite loci were also analysed for mitochondrial control region. The PCR was composed of 12.5 µl PPP Mastermix, 1 µl THR-L (5'-CAATCCCCCGGTCTTGTAACCC-3') and 1 µl DL-H (5'-CCTGAAGTAGAAACCAGATG-3') primers, 8.5 µl of PCR water and 2 µl of DNA. The Bio-Rad thermal cycler was set to:

1. 95°C, 3:00
2. 95°C, 1:00
3. 50°C, 1:00
4. 72°C, 1:00
5. GO TO step 2, 34x
6. 72°C, 10:00
7. 12°C, ∞

The presence of mitochondrial control region in PCR products was verified on 1% agarose gel. Amplified samples were purified by purification kit Invisorb® Fragment CleanUp by STRATEC and dilute according to its concentrations. The mitochondrial DNA was elute into 30 µl of elution buffer. The concentration was measured on NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher).

The reaction contained 2.5 µl purified PCR, 0.5µl TH-L primer and 5.5 µl RNA free water. The concentrates were sequenced at Charles University as the microsatellite loci.

Table 1: Composition of primer mix A.

Primer mix A				
ID	Motif	Motif repeats	Range	Fluorescent label
FH2088	CCCTCTGCCTACATCTCTGC	tetra	93-129	6-FAM
FH2054	GCCTTATTCATTGCAGTTAGGG	tetra	136-172	6-FAM
FH2087	CTGCCACATTCACTGATGC	tetra	224-252	6-FAM
PEZ17	CTAAGGGACTGAACTTCTCC	tetra	220-240	VIC
FH2017	AGCCTCTATAATCACGTGAGCC	tetra	260-276	VIC
FH2001	TCCTCCTCTTCTTTCCATTGG	tetra	132-156	PET
INRA21	ATGTAGTTGAGATTCTCCTACGG	di	87-111	PET
REN169D01	AGTGGGTTTGCAAGTGGAAC	di	199-221	PET
FH2097	CAATGTCGAATTCATGGTG	tetra	260-305	NED
CXX279	TGCTCAATGAAATAAGCCAGG	di	109-133	NED
REN169O18	CACCCAACCTGTCTGTTCT	di	154-170	NED

Table 2: Composition of primer mix B.

Primer mix B				
ID	Motif	Motif repeats	Range	Fluorescent label
FH2096	CCGTCTAAGAGCCTCCCAG	tetra	90-110	6-FAM
FH2137	GCAGTCCCTTATTCCAACATG	tetra	153-180	6-FAM
INU055	CCAGGCGTCCCTATCCATCT	di	190-216	6-FAM
VWF	CTCCCCTTCTCTACCTCCACCTCTAA	hexa	118-178	VIC
FH2161	TCAGCAAGAAACCCTCCAGT	tetra	219-248	VIC
AHTk211	TTAGCAGCCGAGAAATACGC	di	83-101	VIC
CPH5	TCCATAACAAGACCCCAAAC	di	111-119	PET
FH2010	AAATGGAACAGTTGAGCATGC	tetra	217-260	PET
REN64E19	TGGAGAGATGATATCCAAAAGGA	di	139-155	PET
FH2140	GGGGAAGCCATTTTTAAAGC	tetra	99-149	NED
Amelogenin	GTGCCAGCTCAGCAGCCCGTGGT		180; 216	NED

3.4. Data analysing

Allele scoring and binning was performed in Geneious software (Kearse et al. 2012). Only samples amplified on at least 70% of loci were included in further analyses.

The first step was a clustering analysis to determine the origin of the sample. It is important to distinguish wolves from accidentally sampled dogs as the wolves' faeces can be easily mistaken with the dogs'. This analysis was made in Structure 2. 3. 4 software (Pritchard et al. 2000) and New-Hybrids software (Anderson & Thompson 2002). Both are based on Bayesian inference.

Structure is widely used in population genetics and is very useful to reveal the species' population substructures based on allele frequencies. The program assigns each individual to specific cluster (K) and estimates its membership to respective K. As a comparative samples, we used datasets of Slovakian and Polish wolves and feral and pure-breed dogs. Burn-in value was set to 200 000 Markov Chain Monte Carlo (MCMC) iterations, to obtain more accurate results, unbiased by the initial runs of the software. The number of MCMC after burn-in was 800 000 iterations. Number of tested clusters was set from K=1 to K=10. Every K was repeated five times. The results were combined and visualised in Structure Selector (Li & Liu 2018). The analysis in Structure was later used to detect intrapopulation structure of final wolves' dataset. The parameters of the analysis remain unchanged. The membership coefficients for each individual for respective K were displayed in map using ArcGIS (ESRI 011).

To test, if any of the samples could be of a hybrid origin, we used the software New-Hybrids (Anderson & Thompson 2002). The software identifies two pure populations – dogs and wolves, and their F1 hybrids, F2 hybrids and backcrosses. Observed genotypes are divided into these categories according to their genotype pattern. Samples detected of unclear wolf origin were excluded from further analysis.

The Cervus software (Marshall et al. 1998; Kalinowski et al. 2007) uses the likelihood-based approach to compare the genotypes. Firstly, it analysed allele frequencies as a basis for Identity and parentage analysis. Minimum number of matching loci for Identity analysis was set to 10 and fuzzy matching allowed at the level of 5. Samples with a positive match were again checked in Geneious software and the matching genotypes were excluded.

To obtain the parameters of population polymorphism, GenAlEx 6.5 software (Peakall & Smouse 2012) was used. The fixation index, which measures the genetic differentiation within population (Wright 1951), the heterozygosity, the number of effective alleles and the coefficient of inbreeding (Wright 1951) were calculated.

Maximum likelihood estimation of relatedness was done in M-L Relate (Kalinowski et al. 2006). This software compares the genotypes and estimates the rate

of relatedness. The results are divided into four categories: Unrelated, Half Siblings, Full Siblings and Parent/Offspring. The level of confidence was 95% (0.95).

Table 3: A list of k-coefficients for common relationship categories. K_m represents the probability that two individuals share m alleles.

Relationship	k_0	k_1	k_2
Parent/Offspring	0	1	0
Full-siblings	0.25	0.5	0.25
Half-siblings	0.5	0.5	0
First cousin	0.75	0.25	0
Unrelated	1	0	0

The sequences of mitochondrial D-loop were visualised and edited in Geneious software together with sequences of wolves' haplotypes described by Pilot et al. (2010). The known sequences were downloaded from National Centre for Biotechnology Information databases. We used sequences from publication of Hulva et al. (2018) and Jedrzejewski et al. (2012) with accession numbers: MF440675, MF440676, AF344299, AF344300, AF344301, AF344302. Edited sequences were aligned using MAFFT v. 7 (Katoh & Standley 2013). The sequences from our data were assign to known haplotypes.

4. Results

Eight of the samples were excluded due to pattern of hybrid origin according to analyses in Structure (Pritchard et al. 2000) and New Hybrids (Anderson & Thompson 2002). The result from New Hybrids (Anderson & Thompson 2002) is visualised in Fig. 4.

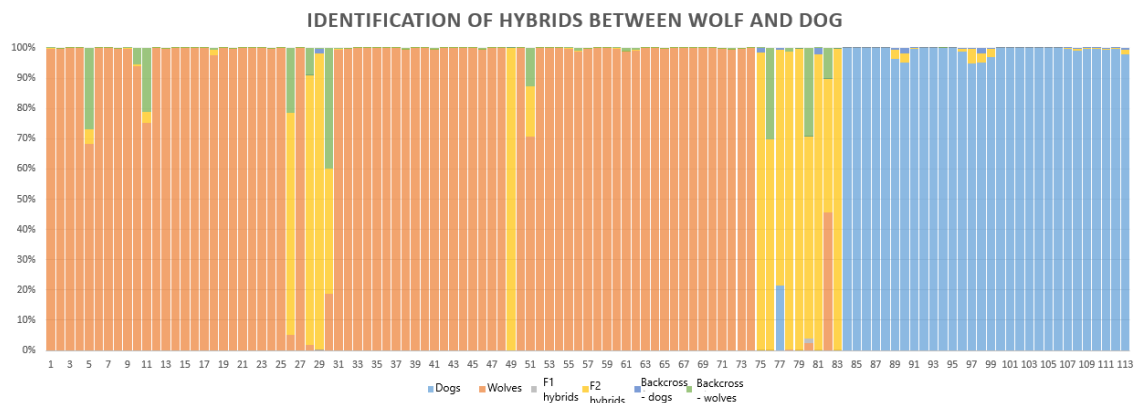


Figure 4: Result from New Hybrids software. Representation of gene pool composition. The samples are divided into described categories of pure populations and possible hybrids. Samples 1-74 are tested samples, 75-83 are feral dogs and 85 – 113 are pure-breed dogs.

The Cervus software (Marshall et al. 1998; Kalinowski et al. 2007) revealed several matching genotypes. Five of the matching genotypes were from Krušné Mountains, two genotypes were from PA Třeboňsko and two genotypes were from PA Broumovsko. In total, six samples were excluded.

The intraspecies population structure is displayed in the Figure 5 and Figure 6 for K=2 and K=3.

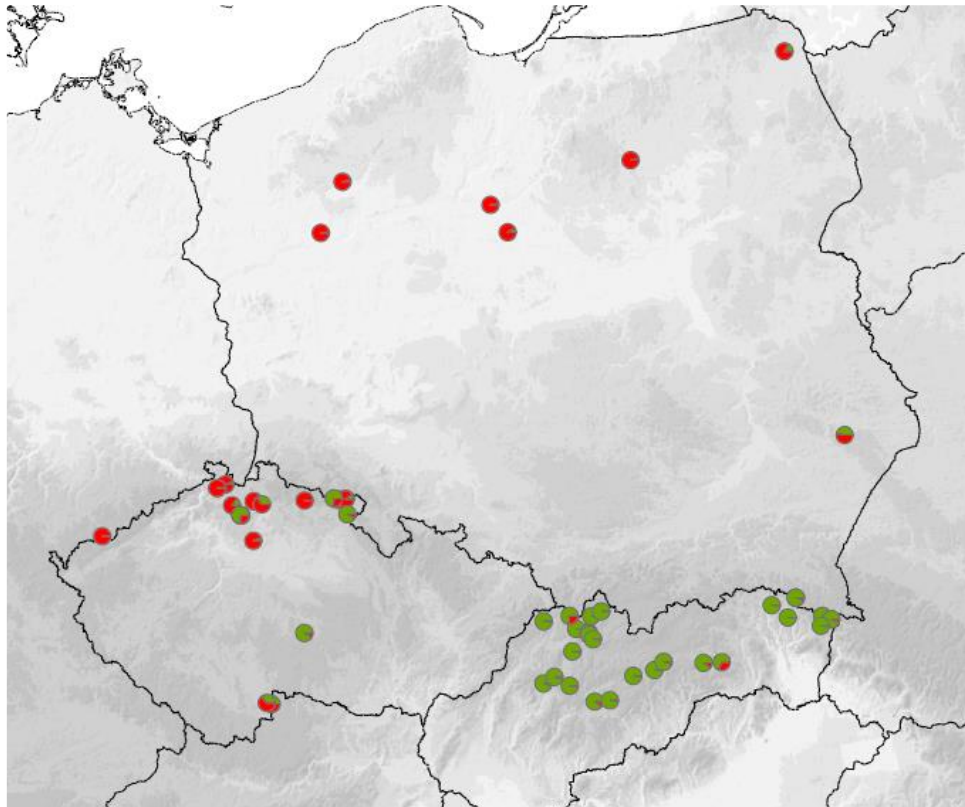


Figure 5: The pie charts representing individuals reflect individual membership to the tested cluster. Analysis is based on 21 microsatellite loci evaluated in Structure software at K=2. The pattern of Carpathian wolves is green and the Lowland population pattern is red. Proportion of colours are equal to membership coefficient from Structure.

The result of K=2 run is indicating the gene flow from Carpathian population into the area of PA Broumovsko.

The K=3 division reflects the uniformity and lower polymorphism of Lowland population. At the same time, the area of PA Broumovsko, PA Třeboňsko and one sample from PA Kokořín represent a separate cluster.

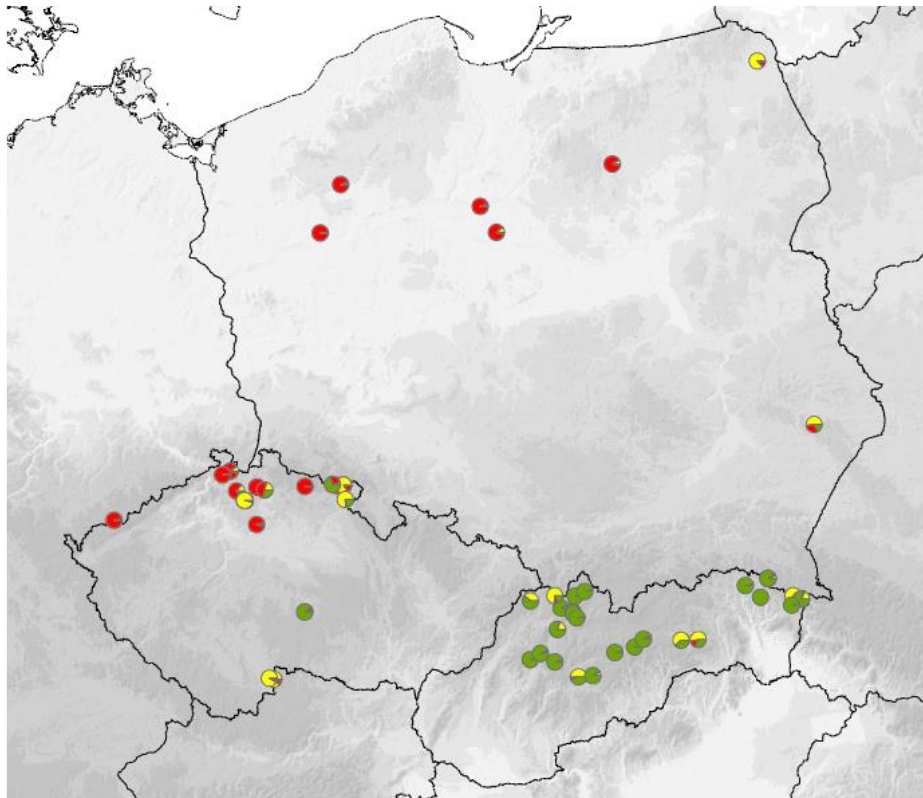


Figure 6: The pie charts representing individuals reflect individual membership to the tested cluster. Analysis is based on 21 microsatellite loci evaluated in Structure software at K=3. The pattern of Carpathian wolves is green and the Lowland population pattern is red. Proportion of colours are equal to membership coefficient from Structure.

In the Figure 7, 8, 9 and 10 individuals from Carpathian population does not show any subpopulation structure. On the other hand, the Lowland population reflect artefacts of substructure.



Figure 7: Result of Structure clustering in K=2. (1) Lowland population, (2) Carpathian population.



Figure 8: Result of Structure clustering in K=4. (1) Lowland population. (2) Carpathian population. It is visible the loss of further genetic substructure within Carpathian population.



Figure 9: Result of Structure software in K=7. The Lowland population is more differentiated than in K=5.

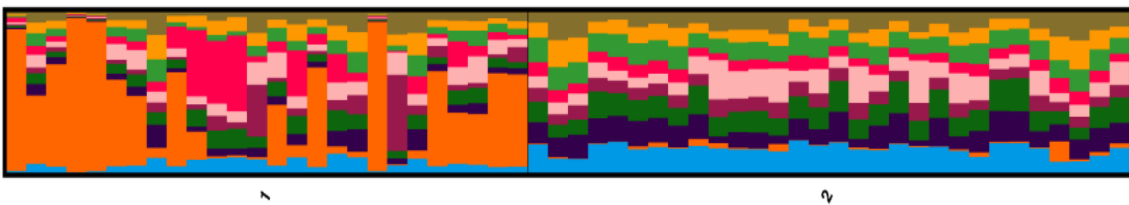


Figure 10: Structure clusters in K=10. Lowland population reflects three subdivisions. The mix, the green colour and red colour.

The Structure Selector evaluated the K=2 and K=3 as the most probable (Fig. 11, Fig. 12).

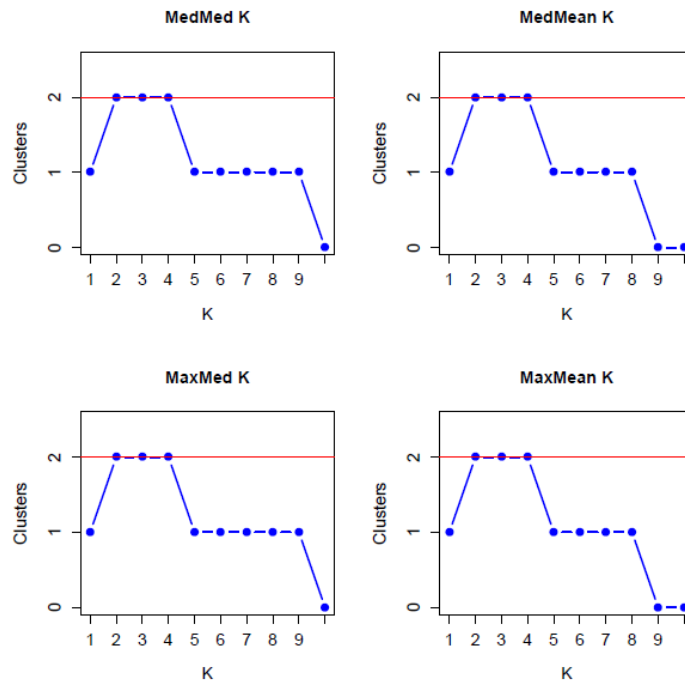


Figure 11: Results of MedMed K and MedMean K suggest the appropriate number of clusters at K=2 according to Li & Liu (2018).

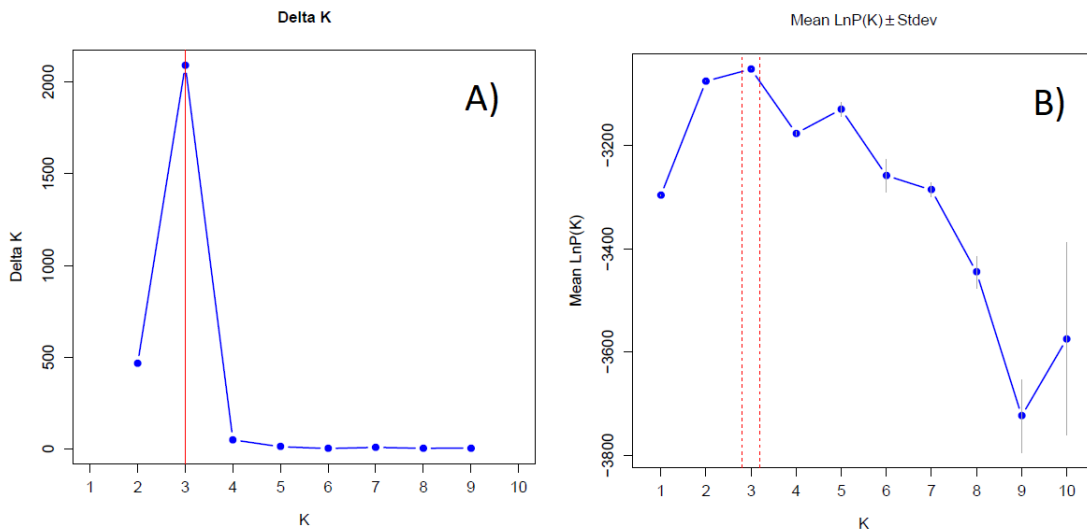


Figure 12: Results of Delta K (A) and LnP(K) (B) according to Evanno et al. (2005). This method uses different algorithm of calculations to detect true number of clusters.

The fixation index for Lowland population reflects average value 0.088 ± 0.033 . In Carpathian population, the parameter is lower. It is 0.038 ± 0.026 . The Lowland population's number of private alleles is 3.327 ± 0.301 , meanwhile the Carpathian

population reflect 3.322 ± 0.233 . The observed heterozygosity is lower than expected in both populations. The Lowland population's expected heterozygosity is 0.655 ± 0.028 , meanwhile the observed is 0.604 ± 0.038 . The values of Carpathian population are similar. Expected heterozygosity is 0.652 ± 0.035 and observed is 0.623 ± 0.036 . Estimated coefficient of inbreeding shows higher value for Lowland population (0.1149) than for Carpathian population (0.0265). The parameters are displayed in Table 4.

Table 4: Parameters of genetic diversity within observed populations. F=fixation index. Ne= Number of private alleles. Ho= Observed heterozygosity. He=Expected heterozygosity. F_{is} =Coefficient of inbreeding.

	F	Ne	Ho	He	F_{is}
LOWLAND	0.088 ± 0.033	3.327 ± 0.301	0.604 ± 0.038	0.655 ± 0.028	0.1149
CARPATHIAN	0.038 ± 0.026	3.322 ± 0.233	0.623 ± 0.036	0.652 ± 0.035	0.0265

Principal coordinates analysis of sampled wolves and dogs is displayed in Figure 13. The genetic variability is explained by 17.86 % by X axis.

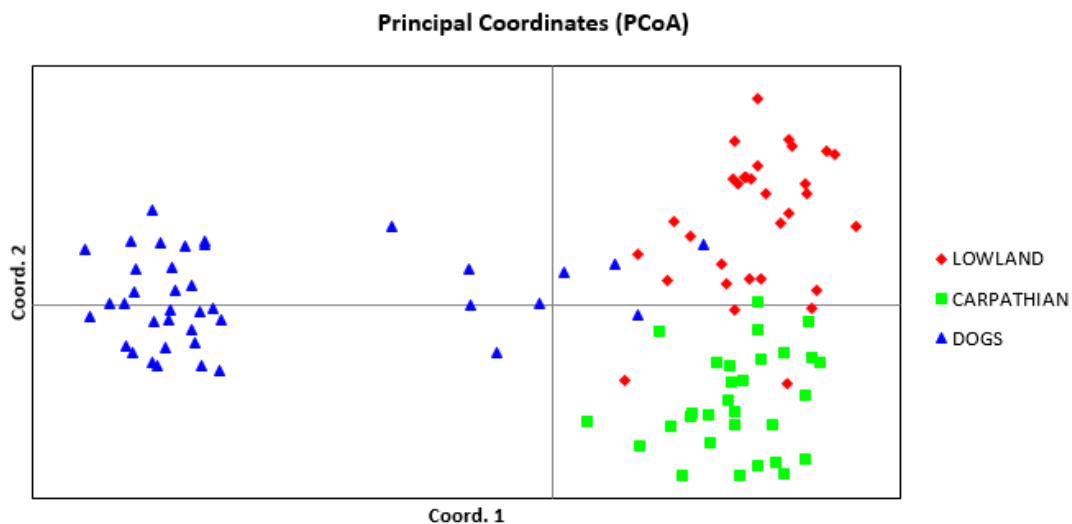


Figure 13: Principal coordinates analysis of wolves and dogs.

The genetic variability of two wolves' populations is explained by 12.4 % by X axis. The result is displayed in Figure 14.

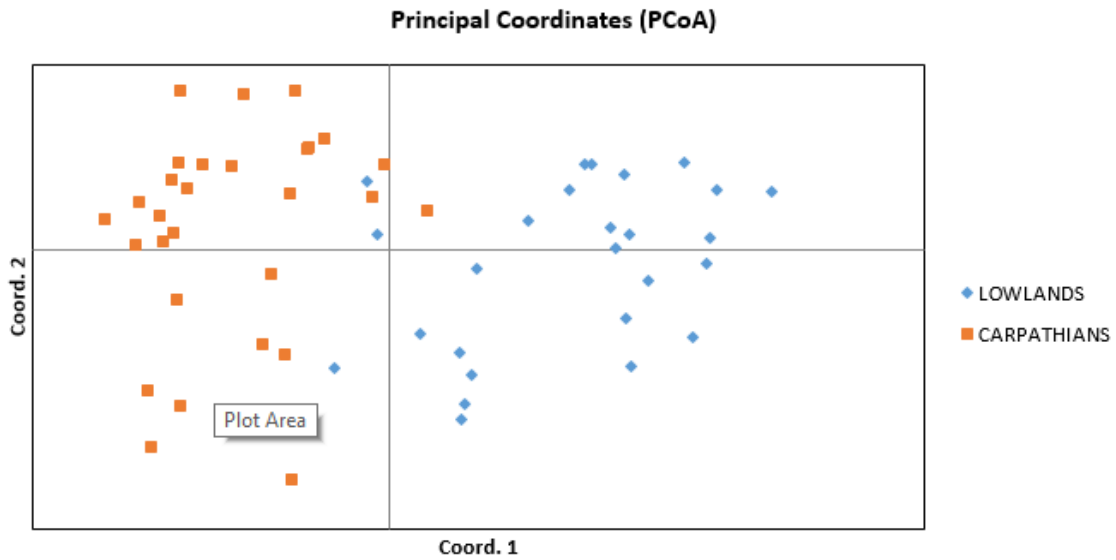


Figure 14: Principal Coordinates Analysis of two wolf populations.

M-L Relate software (Kalinowski et al. 2006) estimated one case of full siblings in PA Broumovsko. The PA Kokořínsko is well sampled and give us deeper insight to the structure of sampled wolves. Within this area, we identified four cases of parent/offspring category, two cases of full siblings and two cases of half siblings. The result is displayed in Table 5.

Table 5: Result of estimated relationships between observed samples.

Sample	Locality	Sample	Locality	Category of Relatedness
G608	PA Kokořín	V647	PA Kokořín	Parent/Offspring
K4	PA Kokořín	G548	PA Kokořín	Parent/Offspring
K4	PA Kokořín	G548	PA Kokořín	Parent/Offspring
K4	PA Kokořín	X1	PA Kokořín	Parent/Offspring
K4	PA Kokořín	V647	PA Kokořín	Full siblings
G548	PA Kokořín	G608	PA Kokořín	Full siblings
V606	PA Broumovsko	G257	PA Broumovsko	Full siblings

Within the dataset, we identified haplotypes: W1, W14, W2, W3 and W6. The pattern of distribution is consistent with Hulva et al. (2018). The geographic distribution of haplotypes is visible in Figure 15.

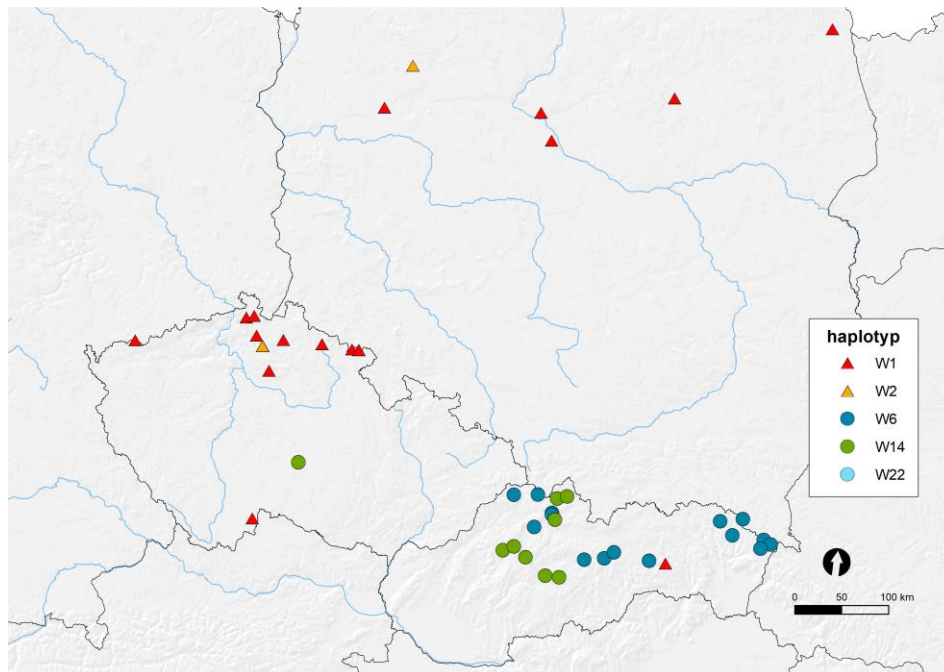


Figure 15: Distribution of haplotypes in Central Europe. The triangles represent haplogroup 1 and circles represent haplogroup 2.

5. Discussion

Wolves as long-distance dispersal animals can cross many artificial barriers (Gula et al. 2009, Theuerkauf et al. 2007) and are able to adapt to various ecosystems and climate conditions (Pilot et al. 2006). The Carpathian population proved the dispersal potential, when genetic analysis revealed the Carpathian wolf hit by car on D1 highway near Jihlava in 2017 (Hulva et al. 2018). In our study, the Bayesian clustering of genotypes at $K=2$ indicates the gene flow between the Carpathian population and PA Broumovsko (Fig. 5, Fig. 6). In the study of Hulva et al. (2018), such a pattern was not observed. One of the possible reasons could be limited sample size from the north Bohemian area in Hulva et al. (2018), which is more likely than the beginning of the gene flow just between these two studies. Future connection of the Lowland and Carpathian population is probable. It was already proved by Hulva et al. (2018) that Lowland genotypes are occurring in Western Carpathians. Authors of the study suggest an establishment of the Lowland individuals within Western Carpathians.

Five samples within Czech Republic (from PA Kokořín, PA Broumovsko and PA Třeboňsko, Fig. 6) genotypes clustered separately at $K=3$. Such a pattern may have similar explanation as the situation in Broumovsko. Unlike the Carpathian and Lowland populations, our dataset did not include another possible source population, which is Italian-Alpine population coming from Austria/Germany (Hulva et al. 2018). Unfortunately, we cannot confirm nor reject the presence of this population in the South Bohemia. If we assume, that samples which cluster separately belong to unsampled population, the observed structure corresponds with the premises stated by Hulva et al. (2018).

In our study, we did not detect any additional haplotypes within Central Europe compared to previous studies, moreover, Bohemian haplotypes matched dominant W1 haplotype of the Lowland population. Due to the fact that males of wolves tend to disperse for longer distances than females (Ballard et al. 1997), the migration pattern may not be visible yet in distribution of maternally inherited mitochondrial haplotypes.

The pattern of haplotype distribution can be considered as steadier than distribution of genotypes.

The research is based on 21 microsatellite loci and one sex-determining gene Amelogenin. Similar studies of wolf geographic distribution and their genetic differentiation in Central Europe used less loci – Hulva et al. (2018) used 18 loci, Czarnomska et al. (2013) used 11 loci. On the other hand, Hulva et al. (2018) used more samples (365) from six Central European countries (Poland, Germany, Czech Republic, Slovakia, Romania, and Austria).

The Lowland population is reported as rather homogenous compared to other wolves' populations (Hulva et al. 2018). Authors describe it as a consequence of more frequent mating between related individuals. Comparison of the parameters of genetic polymorphism (H_o , H_e , F , F_{is} , N_e) between the populations within our study did not follow these results. Most probable explanation is limited amount of samples representing the Carpathian population. We included only several individuals from known subpopulations from Hulva et al. (2018). Such a targeted sampling is biasing description of genetic polymorphism of the whole population.

Positive values of F_{is} were expected in Lowland population due to the short period since the establishment of the pack approximately 35 years ago by few wolves (Andersen et al. 2015). The resultant value ($F_{is}=0.1149$) is defined as moderate (Czarnomska et al. 2013). In a comparison to Hulva et al. (2018), observed values are higher, because the dataset of this thesis contains more samples from the area of northern Bohemia, where the wolves are expected to be related. These findings agree with the estimation of relatedness. The Czech Republic reflects more cases of Parent/Offspring or First Siblings categories, meanwhile the Slovakia reflects only the Half Siblings category in M-L Relate software (Kalinowski et al. 2006). This category was not considered seriously, because it had the accordance at the level of 20%. Due to the limited amount of markers, the conformity could be just a coincidence. To avoid these biases in the future, it is recommended further genomic research, because SNPs can cover wider scope of the gene pool.

This study revealed potential relationships, but the reconstruction is very difficult. It is impossible to obtain the age from the genotype of sampled animal. The construction of the pedigree usually requires the data from other sources such as camera traps. In wolves, such a method is unusable and thus, the succession necessary for the pedigrees, is hard to be deciphered.

Eight samples that were excluded from the study because of the unclear origin, should be included in more detailed study focused on the hybridization and using more markers. We did not aim to test it in this thesis because of the complexity of the process. History between dogs and wolves is rather short, moreover their genomes did admix several times after the domestication process had started. From that point of view, we see 21 microsatellite markers as not enough powerful, to further test the hybridization.

Personally, I recommend to deepen the cooperation between Central European countries and their conservation associations. As the animals do not respect the borders of the countries, closer communication between neighbouring countries is necessary. Further cooperation could bring the complex information about the dispersion and ecology of wolves.

6. Conclusions

The continuity of wolf populations in Europe is increasing from the west to the east. The increasing trend of European wolf populations is a reflection of the legal protection and sociological changes. Recently, we noticed several connections of distinct populations, such as Alpine – Italian with Dinaric-Balkan.

We detected the genotype pattern of dispersion from Carpathian Mountains to the area of PA Broumovsko located in the northeast of the Bohemia. After the wolf female hit by car on D1 highway in Czech Republic, it is another case of dispersion of the Carpathian wolf further to the Czech Republic.

In the future studies, the Czech population should be compared with Alpine population to obtain more precise results.

Wolves from the area of Bohemia are densely sampled and several observed genotypes from north Bohemia are highly related, which is an assumption for further dispersion in different regions of Czech Republic or neighbouring countries. Positive values of coefficient of inbreeding suggest increased homogeneity of Lowland population.

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Appendices

List of the Appendices:

Appendix 1: Table of isolated samples.....	I
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Appendix 1: Table of isolated samples.

ID	Year	Month	Day	Locality	Area	N	E	type	isolation
K024	2017	9	17	Abertamy	Krušné Mountains			faeces	13.11.2017
K020								faeces	13.11.2017
V1036	2016	9	18	Kraví potok	Krušné Mountains			faeces	13.11.2017
V962	2016	10	24		Kokořínsko			faeces	13.11.2017
G364								faeces	13.11.2017
V477	2016	10	3	Hričovec	Javorníky			faeces	13.11.2017
G324								faeces	13.11.2017
V674								faeces	13.11.2017
V478	2016	1	29	Velká Rača	Kysucké Beskydy	49.405931	18.973211	faeces	13.11.2017
S014	2017	1	29	Božídarské	Krušné			faeces	13.11.2017

				rašeliniště	Mountains				
FM1								faeces	13.11.2017
FM2								faeces	13.11.2017
K014								faeces	20.11.2017
K022								faeces	20.11.2017
KDF1								faeces	20.11.2017
K015								faeces	20.11.2017
G354	2017	2	5	Podbezdězí	Ralsko	50.545963	14.783336	faeces	20.11.2017
V790								faeces	20.11.2017
V792								faeces	20.11.2017
V699	2016	2	25	Kyčeka	Kysucká highland			faeces	20.11.2017
S017								faeces	20.11.2017
V944								faeces	20.11.2017
F448	2017	4	12	Cínovec	Krušné			faeces	20.11.2017

					Mountains				
G547	2017	10	22	Báně	Podbezdězí	50.564480	14.726860	faeces	4.12.2017
G548	2017	10	22	Báně	Podbezdězí	50.563520	14.712660	faeces	4.12.2017
V583	2016	7	21	Bumbálka	Beskydy Mountains			faeces	4.12.2017
V713	2016	11		Balnica	Bukovské highlands	49.185036	22.214418	faeces	4.12.2017
G490	2017	7	26	Břehyně	Podbezdězí	50.568220	14.697630	faeces	4.12.2017
G514	2017	9	3	Hradčany - Všivá rokle	Podbezdězí	50.625078	14.667921	faeces	4.12.2017
G489	2017	7	26	Hradčanské stěny	Ralsko	50.609880	14.705600	faeces	4.12.2017
G532	2017	10	15	Břehyně	Podbezdězí	50.567660	14.727220	faeces	4.12.2017
G517	2017	9	2	Břehyně - Flešl	Podbezdězí	50.567277	14.732449	faeces	4.12.2017
K021								faeces	4.12.2017

JH								faeces	4.12.2017
V271	2017	9	14	Hricovcom	Javorníky			faeces	13.12.2017
KL01								faeces	13.12.2017
K012	2017	8	5	Mečová	Beskydy			faeces	13.12.2017
V564	2017	8	10	Tichá dolina	Western Carpathians			faeces	13.12.2017
G537	2017	10	14	Pramen Chomutovky	Krušné Mountains	50.490880	13.184040	faeces	13.12.2017
LK2								faeces	13.12.2017
F515	2017	9	20	Zlatý vrch	Podbezdězí			faeces	13.12.2017
F516	2017	9	20	Mariánská cesta	Podbezdězí			faeces	13.12.2017
G350	2017	1	21		Malá Fatra			faeces	13.12.2017
ALB1								faeces	13.12.2017
V276	2015	7	28	Velká Rača	Kysucké Beskydy			faeces	13.12.2017

V800	2017				Bieszczady	49.112051	22.402531	faeces	20.12.2017
V758								faeces	20.12.2017
V978	2016	9	24	u Flesla	Kokořínsko			faeces	20.12.2017
G619	2017	10	23	Pramen Chomutovky	Krušné Mountains	50.553041	13.257613	faeces	17.1.2018
G695	2018	1	7	Malý buk	Lužické Mountains	50.804090	14.555620	faeces	17.1.2018
G613	2017	11	25	Srní louže	Podbezdězí	50.552450	14.731570	faeces	17.1.2018
G608	2017	11	25	Báně	Podbezdězí	50.562930	14.731970	faeces	17.1.2018
G591	2017	11	25	Břehyně	Podbezdězí			faeces	17.1.2018
G609	2017	11	25	Mariánská cesta	Podbezdězí	50.558550	14.731460	faeces	17.1.2018
G694	2018	1	7	Malý buk	Lužické Mountains	50.803010	14.558010	faeces	17.1.2018
G667	2017	12	19	Černý močál	Podbezdězí	50.570450	14.751640	faeces	17.1.2018

G692	2018	1	6	Novinská hůrka	Ralsko	50.681660	14.877530	faeces	17.1.2018
G-KH								faeces	17.1.2018
G584	2017	11	25	Chrastná - Osečná	Ralsko			faeces	17.1.2018
G618	2017	11	18	Kovářská, Velký špičák	Krušné Mountains			faeces	17.1.2018
G519								faeces	17.1.2018
G602	2017	11	25	Fleslovo jezíčko	Podbezdězí	50.567120	14.733380	faeces	17.1.2018
G617	2017	11	8	Pramen Chomutovky	Krušné Mountains	50.494636	13.215074	faeces	17.1.2018
G603	2017	11	25	Báně	Podbezdězí	50.565200	14.732210	faeces	17.1.2018
K007	2017				Bukovské Mountains			faeces	22.1.2018
G626	2017	11	25	Štica-cesta	Javorníky			faeces	22.1.2018
V1043	2017	10	26	Javorské	Kysucké			faeces	22.1.2018

					Beskydy				
G569	2017	11			Javorníky			faeces	22.1.2018
S045	2017	12	29	Vraní hory	Broumovsko			faeces	22.1.2018
G680	2018	1	1	Vraní hory	Broumovsko			faeces	22.1.2018
S048	2017	12	29	Chvaleč	Broumovsko			faeces	22.1.2018
V796	2017				Bieszczady	49.155994	22.361353	faeces	22.1.2018
G674	2017	12	10	Zábrdský kopec	Ralsko	50.683720	14.930520	faeces	22.1.2018
G562	2017	10	28	U spálené hájenky	Břehyně	50.570270	14.751612	faeces	22.1.2018
G673	2017	12	10	Holičský vrch	Ralsko	50.680670	14.917970	faeces	22.1.2018
V799	2017				Bieszczady	47.175918	22.346762	faeces	23.1.2018
V978	2016	9	24	u Flesla	Kokořínsko			faeces	23.1.2018
V758								faeces	23.1.2018

V745								faeces	23.1.2018
AZ001								faeces	23.1.2018
V1007	2017	4	1	Břehyně	Kokořínsko- Máchův kraj	50.567697	14.728237	faeces	23.1.2018
V761								faeces	23.1.2018
G616	2017	11	26	Jáchym	Šluknovsko	51.030740	14.343430	faeces	21.2.2018
K013	2017	10	15	Ptačí vrch	Šluknovsko	50.966513	14.450843	faeces	21.2.2018
G735	2018	1	26	Báně	Podbezdězí	50.568120	14.725350	faeces	21.2.2018
G760	2018	1	30	Báně	Podbezdězí	50.565110	14.732280	faeces	21.2.2018
G768	2018	2	2	V Borůvčí	Hradčanské stěny	50.614430	14.680320	faeces	21.2.2018
G759	2018	1	30	Báně	Podbezdězí	50.565080	14.732240	faeces	21.2.2018
S037	2018	2	8	Vraní hory	Broumovsko			faeces	11.4.2018
S035	2018	1	7	Vraní hory	Broumovsko			faeces	11.4.2018
S034	2018	2	8	Vraní hory	Broumovsko			faeces	11.4.2018

G948	2018	2	8	Vraní hory	Broumovsko			faeces	11.4.2018
S055	2018	1	20	Horní Adršpach	Broumovsko	50.639101	16.069462	faeces	11.4.2018
S050	2018	1	6	Vraní hory	Broumovsko	50.648721	16.027803	faeces	low quality
S030	2018	1	27	Skorkov, D1	Bohemian- Moravian Highlands	49.501247	15.484854	faeces	11.4.2018
S051	2018	1	7	Vraní hory	Broumovsko	50.651335	16.019086	faeces	11.4.2018
G733								faeces	11.4.2018
G877								faeces	11.4.2018
G842	2018	3	4	Velká Tisová	Lužické Mountains	50.837350	14.539850	faeces	11.4.2018
G841	2018	3	4	Velká Tisová	Lužické Mountains	50.841360	14.543280	faeces	11.4.2018
G949	2018	2	22	Vraní hory	Broumovsko			faeces	11.4.2018
K101	2018	1	6	Zlatý kopec	Krušné M.			faeces	25.4.2018

K017	2018	2	17	Rýžovna	Krušné Mountains			faeces	25.4.2018
G896	2018	3	19	Stará plantáž	Ralsko	50.577930	14.765300	faeces	25.4.2018
G961	2018	3	28	Bouřňák	Krušné Mountains			faeces	25.4.2018
G897	2018	3	19	Stará plantáž	Ralsko	50.577660	14.765650	faeces	25.4.2018
G853	2018	3	3	Štica	Javorníky			faeces	25.4.2018
K093	2018	2	10	Jaworova	Broumovsko	50.653981	16.007800	faeces	25.4.2018
G947	2018	2	8	Vraní hory	Broumovsko			faeces	25.4.2018
K085	2018	3	10	Karlów	Broumovsko	50.475111	16.351577	faeces	25.4.2018
S038	2018	2	8	Vraní hory	Broumovsko			faeces	25.4.2018
K063	2018	3	18	Slavný	Broumovsko	50.523737	16.300934	faeces	25.4.2018
G852	2018	3	3	Štica	Javorníky			faeces	25.4.2018

S759	2018	8	28	Široké Blato	Třeboňsko	48.912660	14.989900	faeces	1.10.2018
G1198	2018	7	29	U Trojzubce	Ralsko	50.605500	14.722080	faeces	1.10.2018
S290	2018	3	7	Široké Blato	Třeboňsko	48.905510	14.975890	faeces	1.10.2018
S319	2018	6	19	Široké Blato	Třeboňsko	48.908231	14.991070	faeces	1.10.2018
G642								faeces	1.10.2018
G1282	2018	9	22	Hřelec	Lužické Mountains	50.833270	14.505760	faeces	1.10.2018
V608	2017	3	5	Minčol	Oravská Magura			faeces	10.9.2018
G299	2016	10	8		Kokořínsko			faeces	10.9.2018
G831	2017	2	12	Púpov	Kysucká highland			faeces	10.9.2018
G721	2018	1	6	Krušné Hory	Zlatý kopec			faeces	10.9.2018
CLL1	2017	2	15	Šrámková	Malá Fatra	49.113260	19.000687	faeces	10.9.2018
CLL2	2017	2	15	Šrámková	Malá Fatra	49.113420	19.060927	faeces	10.9.2018

KK01	2018	10	18		Kokořínsko	50.629438	14.656233	buccal swab	19.10.2018
G1426	2018	11	10	Rybí loučky	Jizerské Mountains	50.848850	15.339990	faeces	13.1.2019
G1470	2018	11	28	Černý vrch	Jizerské Mountains	50.825100	15.306270	faeces	13.1.2019
G1479	2018	10	3	Široké blato	Třeboňsko			faeces	13.1.2019
G1480	2018	10	3	Široké blato	Třeboňsko			faeces	13.1.2019
K065	2018	12	6	Vysoká	Vsetínské highlands	49.402179	18.350768	faeces	13.1.2019
K086	2018	5	5	Vraní hory	Broumovsko			faeces	13.1.2019
G1390	2018	10	27	Komáří vrch	Krušné Mountains			faeces	1.3.2019
K055	2019	1	21	Ruprechtice	Broumovsko	50.661955	16.258463	faeces	1.3.2019
G1514	2018	12	27	Valtengrund	Šluknovsko	51.065840	14.275440	faeces	1.3.2019
G1464	2018	11	24	Mlýny	Lužické Mountains	50.814610	14.479930	faeces	1.3.2019

K107	2018	10	27	Rusová	Krušné Mountains			faeces	1.3.2019
G1468	2018	11	24	Mlýny	Lužické Mountains	50.818290	14.508490	faeces	1.3.2019
G1423	2018	10	31	Břehyně	Ralsko			faeces	4.3.2019
K023	2018	10	27	Komáří vrch	Krušné Mountains	50.481365	13.181336	faeces	4.3.2019
G1556	2018	11	10	Široké blato	Třeboňsko	48.917170	14.984800	faeces	4.3.2019
G1565	2018	12	19	Hřebec	Lužické Mountains	50.839310	14.511630	faeces	4.3.2019
G1409	2018	11	4	Břehyně	Ralsko	50.576120	14.729590	faeces	4.3.2019
G1469	2018	11	24	Sokol	Lužické Mountains	50.829130	14.529540	faeces	4.3.2019
K025	2018	10	27	Božidarské raš.	Krušné Mountains	50.406930	12.884900	faeces	4.3.2019
K056	2018	10	27	Pram.	Krušné	50.475795	13.180195	faeces	4.3.2019

				Chomutovky	Mountains				
K097								faeces	4.3.2019
G1385	2018	10	26	Veselí	Ralsko	50.633100	14.638660	faeces	4.3.2019
G1437	2018	11	12	Veselí	Ralsko	50.631650	14.647690	faeces	4.3.2019
G1438	2018	11	12	Nad Kraví roklí	Ralsko	50.616560	14.677010	faeces	4.3.2019
G1445	2018	11	17	Zhůřský potok	Šumava National Park	49.170750	13.342270	faeces	4.3.2019
G1455	2018	11	22	Břehyně - Báně	Ralsko	50.571130	14.703290	faeces	4.3.2019
G1472	2018	12	1	Studničkův les	Ralsko	50.536836	14.778440	faeces	4.3.2019
G1473	2018	12	1	U Spálené hájenky	Ralsko	50.571140	14.756030	faeces	4.3.2019
G1542	2019	1	21	Javoří hory- Ruprechtice	Broumovsko	50.660057	16.264665	faeces	4.3.2019

G1566	2019	1	13	Pěnkavčí vrch	Lužické Mountains	50.855300	14.607720	faeces	4.3.2019
G1380	2018	10	25	Hradčany - U Obrázku	Ralsko	50.615730	14.663680	faeces	4.3.2019
G1458	2018	11	22	Břehyně - Báně	Ralsko	50.570200	14.711590	faeces	4.3.2019
G1465	2018	11	24	Mlýny	Lužické Mountains	50.814530	14.480310	faeces	4.3.2019
CK	2019	2	23	Česká Kamenice				tissue	28.2.2019