HUNGARIAN UNIVERSITY OF AGRICULTURE- AND LIFE SCIENCES

DEVELOPMENT OF GENETIC AND GENOMIC METHODS FOR POPULATION GENETICS OF HUNGARIAN PREDATORY MAMMAL SPECIES

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Péter Árpád Fehér
Gödöllő
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Doctoral School

Name: Animal Biotechnology and Animal Sciences

Discipline: Animal Sciences

Head: **Dr. Miklós Mézes**
D. Sc. Professor, member of HAS
MATE, Institute of Physiology and Nutrition
Department of Feed Safety

Supervisor(s): **Dr. Viktor Stéger**
Head of the Department, Senior Research Fellow
MATE, Institute of Genetics and Biotechnology
Department of Genetics and Genomics

Dr. László Szemethy
Professor
University of Pécs
Faculty of Science, Institute of Biology

………………………………..
Head of Ph.D. School

Dr. Miklós Mézes,
member of HAS

……………………………
Supervisors

Dr. Viktor Stéger,
Senior Research Fellow

……………………………

Dr. László Szemethy,
Professor

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1. INTRODUCTION AND AIMS

1.1 Introduction

Carnivores, but especially large carnivores are among the most controversial and challenging groups of species to conserve in our modern and crowded world (Chapron et al. 2014). Their protection in advanced countries has strict criteria not just in terms of habitat and prey availability, but also for local communities and stakeholders, e.g. farmers and game managers (Linnell et al. 1999, Berger 2006).

These species are important members of ecosystems, as they are mostly higher up in the ecosystem or are apex carnivores. Therefore, they can regulate the populations of other organisms, stabilise the community and improve the quality of prey species populations (Heltai & Szemethy 2010). Also, carnivores can be very good indicators, because their role can characterise the state of the ecosystem. Changes in the quantity and quality of prey populations affect carnivores, can regulate their numbers, so the two populations interact with each other (Török & Fodor 2002). Nowadays several large carnivore populations – such as those of the grey wolf and the brown bear – are on the rise in Europe (Deinet et al. 2013), mainly due to conservation programmes, legal protection and habitat rehabilitation (Chapron et al. 2014).

It has always been difficult for biologists to study rare animal species, such as carnivores (Long et al. 2008), because these species are difficult to observe due to their cryptic lifestyle. Appropriate monitoring methods for these species use non-invasive techniques (Boitani & Powell 2012), some of which are low-budget (Heurich et al. 2012). Common methods for detecting the presence of mammal species include tracking and faecal or urine collection (Liebenberg 1990; MacKay et al. 2008; Schwartz & Monfort 2008), acoustic monitoring (Comazzi et al. 2016), camera trapping (Meek et al. 2014), and

hair collection (Kendall & McKelvey 2008). With these monitoring methods, identification is usually only possible up to Family level (Kendall & McKelvey 2008, Heinemeyer et al. 2008). For species and individual identification, or population characteristics (bottleneck, kinship, density) genetic methods are needed. Among these methods, faecal and urine analyses and hair analyses also allow genetic monitoring (Kendall & McKelvey 2008).

1.2. Aims

The Bükk National Park Directorate, the Institute of Wildlife Management and Nature Conservation of the Hungarian University of Agricultural and Life Sciences (previously Institute of Wildlife Conservation of the Szent István University), and the Institute of Genetics and Biotechnology (previously Agricultural Biotechnology Institute of the National Agricultural Research and Innovation Centre) have been collaborating on genetic monitoring of protected carnivores since 2014. That is how a protocol has been developed for genetic monitoring of carnivore species such as felids (wildcat, Eurasian lynx), and canids (grey wolf, golden jackal) (Fehér et al. 2017).

In my thesis my goal was to:

1. optimize the microsatellite markers used in the international literature for wild canids and felids mainly collected by non-invasive methods;
2. explore the origin of wolves appearing in the North Hungarian Mountains, using grey wolf samples collected primarily in the field in Slovakia and Hungary, and captive wolf samples;
3. explore the kinships of wolves appearing in the North Hungarian Mountains;
4. identify the species of Eurasian lynx by genetic methods, determine the minimum number of individuals and the sex ratio.

5. In view of the hybridisation process previously detected in the Hungarian wildcat population, I aimed to assess the genetic diversity of the wildcat population in the North Hungarian Mountains, in particular to detect possible hybridisation with domestic cats. In case of uncertainty I aimed to test the reliability of the primers used.

2. MATERIALS AND METHODS

2.1. Sampling and DNA preparation

Microsatellite markers adapted from the international literature were used for genotyping and were optimized under multiplex conditions. Optimisation was performed on dog, cat, captured wolf, wildcat, and Eurasian lynx samples. Samples collected in nature came from the Bükk National Park Directorate, the Danube-Ipoly National Park Directorate, and the Hortobágy National Park Directorate in Hungary, and from the Tatra Mountains in Slovakia. I investigated a total of 117 grey wolf samples, 10 Eurasian lynx samples and 27 field samples of wildcat. Whole genomic DNA was extracted from field and reference samples of faeces, urine, hair, bone, tissue, blood and muscle. Samples were stored at – 20 °C until use.

2.2. Population genetic studies using STR markers

Canine samples were genotyped at 14 microsatellite loci, and feline samples at 21 microsatellite loci which were first optimized under multiplex conditions. Sex was determined using the amelogenin gene (Yan et al. 2013, Pilgrim et al. 2005). Optimized multiplex PCRs were set up in a total volume of 25 µl, containing 2 x QIAGEN Multiplex Master Mix (QIAGEN GmbH, Germany), 45-240 ng of template DNA, each primer in optimum concentration (10 µM), and filled up with water. I verified the success of the reactions by agarose gel electrophoresis in 1.5% agarose gel using the GeneRuler 100 bp DNA Ladder (Thermo Scientific, USA). Amplified PCR products were separated on an ABI Prism 3100 Genetic Analyser, carried out by BIOMI Ltd. The PeakScanner ver. 1.0 (Applied Biosystems, USA) was used to analyse electropherograms and score allele sizes. The electropherograms were processed using the PeakScanner (Applied

Biosystems, USA), and the genotypes of the samples were recorded in a Microsoft Excel table. Based on the table, I converted the genotype data to the various formats required using the GenAlEx Excel extension (Peakall & Smouse 2012) and CONVERT v.1.31 softwares (Glaubitz 2005). Null alleles and scoring errors were detected using the MICRO-CHECKER software (Van Oosterhout et al. 2004)

2.2.1. Grey wolf

I used GenAlEx ver. 6.5 (Peakall & Smouse 2012) to estimate allele frequency by locus and population, the number of alleles (Na), the number of effective alleles (Ne), and observed and expected heterozygosity (H_O, H_E) values. Allelic richness (AR) values were computed with FSTAT ver. 2.9.3.2 (Goudet 1995). The Bayesian clustering method implemented in STRUCTURE ver. 2.3.4 (Pritchard et al. 2000) was used to infer the most probable number of genetic clusters without *a priori* definition of populations to assess potential admixture between Hungarian wolves and other wolves and dogs. I estimated the number of clusters (K) by calculating the second-order rate of change in log-likelihood values (ΔK) in STRUCTURE Harvester ver. 0.6.94 (Earl and vonHoldt 2012), as well as the four supervised estimators of K from threshold values (Puechmaille 2016) in StructureSelector (Li and Liu 2018). I also used discriminant analyses of principal components (DAPC) implemented in adegenet ver. 2.1.1 (Jombart 2008), which identifies clusters of individuals without using any population genetic model (Jombart et al. 2008). I used the find.clusters() function for the identification of the optimal number of clusters based on the Bayesian information criterion (BIC). The adegenet package was run with R ver. 4.0.1 (R Core Team 2018). I detected the direction of differentiation with the diveRsity package (Keenan et al. 2013) and used this information to infer relative migration between pairs of

populations (Sundqvist et al. 2016). In order to identify parental and kinship relations among Hungarian wolves and to define family groups (packs), we used the “parentage assignment” package Colony2 (Jones and Wang 2010).

2.2.2. Felids: Eurasian lynx and wildcat

I used GenAlEx ver. 6.5 (Peakall & Smouse 2012) to estimate allele frequency by locus and population, the number of alleles (Na), the number of effective alleles (Ne), observed and expected heterozygosity (H_O, H_E) values and the Shannon-Weaver diversity index. For the cluster analyses I used the STRUCTURE ver. 2.3.4 (Pritchard et al. 2000) software. I estimated the number of clusters (K) by calculating the second-order rate of change in log-likelihood values (ΔK) in STRUCTURE Harvester ver. 0.6.94 (Earl and vonHoldt 2012). Individuals were considered to belong to a cluster (domestic cat or wildcat) if the assignment probability ($q^{(i)}$) was ≥ 0.75. Individuals with $q^{(i)} < 0.75$ but higher than 0.25 were classified as hybrids (domestic cat x wildcat hybrids). To investigate the power of STR markers to detect domestic cat and wildcat hybridisation, I generated domestic cat, wildcat and hybrid genotypes using Hybridlab (Nielsen et al. 2006). To generate genotypes, I used complete empirical microsatellite genotypes (without missing loci), that can be assigned to a parental species with an assignment probability ≥ 0.75 based on STRUCTURE analyses. I generated a total of 240 individuals of each of the following categories: parentals, F1s, F2s, and first and second backcrosses to either parental species (30 generated genotypes per group). I performed another STRUCTURE analysis with the parental and hybrid genotypes generated by the software.

3. RESULTS

3.1. Grey wolf

3.1.1. Genetic diversity

Of the 86 field samples of predicted grey wolves in Hungary, 35 samples were identified as full genotypes, and 22 samples as partial genotypes. I used 15 samples from Slovakia, 9 samples of captive wolves, and 14 samples of dogs for the studies. I was able to identify the complete genotypes of these samples. Diversity values per loci and per groups can be found in Table 1.

Table 1: Polymorphism of 14 autosomal microsatellite loci in Hungarian and Slovakian free-ranging wolves.

	Hungarian wolves							Slovakian wolves						
STR	N	N_A	H_o	H_E	HWE	PIC	I	N	N_A	H_o	H_E	HWE	PIC	I
c2001	25	4	0.64	0.73	***	0.62	1.21	15	5	0.73	0.73	ns	0.69	1.41
c2054	25	6	0.92	0.71	ns	0.68	1.43	15	5	0.80	0.71	ns	0.67	1.42
FH2538	25	7	0.60	0.78	ns	0.67	1.44	15	8	0.87	0.78	ns	0.76	1.78
PEZ3	25	4	0.52	0.71	ns	0.6	1.18	15	6	0.60	0.71	***	0.67	1.43
PEZ8	25	6	0.72	0.54	ns	0.69	1.50	15	4	0.47	0.54	ns	0.49	0.99
PEZ19	25	3	0.56	0.29	ns	0.46	0.88	15	3	0.20	0.29	ns	0.26	0.53
FH2088	25	6	0.68	0.73	***	0.68	1.50	15	5	0.73	0.73	ns	0.68	1.41
PEZ02	25	5	0.60	0.66	ns	0.63	1.27	15	4	0.40	0.66	*	0.6	1.19
FH3377	25	6	0.68	0.68	ns	0.62	1.37	15	5	0.80	0.68	**	0.63	1.29
FH2010	25	6	0.36	0.64	***	0.47	1.06	15	5	0.53	0.64	ns	0.59	1.23
FH2004	25	8	0.44	0.76	***	0.62	1.41	15	7	0.73	0.76	ns	0.73	1.61
FH2107	25	11	0.72	0.87	*	0.87	2.23	15	12	0.80	0.87	ns	0.86	2.24
FH2309	25	5	0.40	0.76	***	0.47	1.01	15	6	0.80	0.76	ns	0.72	1.56
FH3313	25	11	0.60	0.80	***	0.78	1.93	15	8	0.80	0.80	ns	0.77	1.82
Overall	-	6.29	0.60	0.68	-	0.63	1.39	-	5.93	0.66	0.69	-	0.65	1.43

Number of individuals (N), number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, Hardy-Weinberg equilibrium (HWE),

Polymorphism Information Content (PIC), Shannon's Information Index (I). ns = non-significant; * = $p<0,05$; ** = $p<0,01$; *** = $p<0,001$.

3.1.2. Relationships between Hungarian individuals

The program Colony2 detected several siblings and offspring-parent relationships among the sampled Hungarian wolves. Based on the "Best Cluster" result, three generations were identified for a wolf pack in the Bükk Mountains. The sex determination of these individuals revealed four males and one female.

3.1.3. Genetic structure

The program STRUCTURE detected the highest average log-likelihood values for six genetic clusters (K = 6), and the second-order rate of change in log-likelihood values was the highest for two genetic clusters, K = 2 (Figure 1/A, 1/B). In this case, free-ranging Slovakian and Hungarian wolves clustered together forming one group, whereas dogs and captive wolves formed the other cluster (Figure 1/D). The four supervised estimators of Puechmaille indicated the presence of four clusters throughout all threshold values, K = 4 (Figure 1/C). In this case, dogs and captive wolves formed two distinct groups and separated well from the other samples, Slovakian wolves formed another group clustered with some Hungarian samples and the rest of the Hungarian samples formed the fourth group (Figure 1/D).

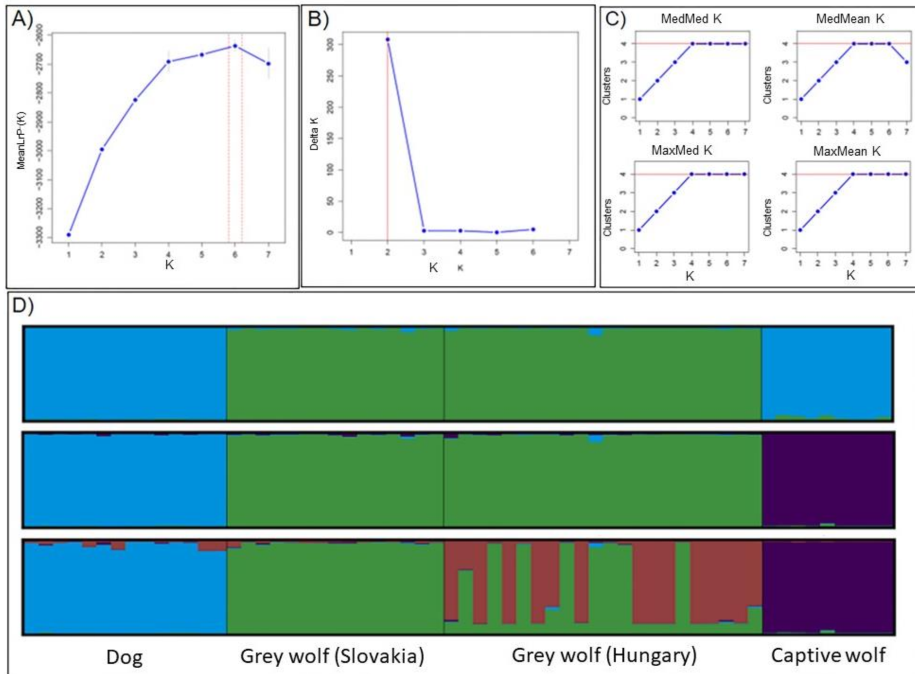

Figure 1: The results of the Bayesian clustering of free-ranging wolves in Slovakia and Hungary, captive wolves and dogs. A) The mean log-likelihood values for each value of the number of clusters (LnP(K). B) The probability of the models according to cluster size based on the second-order rate of change in log-likelihood values (DeltaK). C) The optimal number of clusters based on supervised estimators (MedMed K, MedMean K, MaxMed K, MaxMean K). D) Bar plot of membership probabilities from K = 2 to K = 4.

DAPC also showed the lowest BIC scores for K = 4 (Figure 2/A). Similarly to the results from STRUCTURE, dogs and captive wolves formed two distinct groups and separated well from the other samples. Free-ranging wolves formed two clusters that corresponded to Slovakian and Hungarian samples, but again, some Hungarian samples clustered with Slovakian wolves (Figure 2/B). Using diveRsity, a significant relative movement was found only between Slovakian and Hungarian free-ranging wolves. All other possible migration rates were small and non-significant. Migration between free-

ranging wolves seemed to be unidirectional: from Slovakia to Hungary (Figure 2/C).

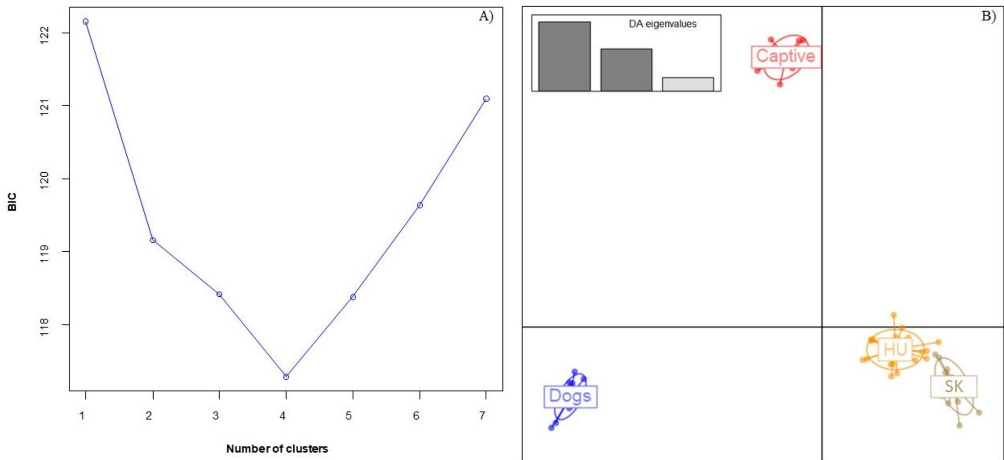

Figure 2: Discriminant analysis of principal components (DAPC) to identify clusters of individuals without using a population genetic model. A) Bayesian information criterion (BIC) according to the number of clusters in the DAPC. The most likely number of clusters is where BIC is lowest. B) DAPC scatter plot showing genetic separation of free-ranging wolves (HU, SK), dogs and captive wolves; DA eigenvalues are shown in the upper left corner.

3.2. Eurasian lynx

Of the reference samples, in one of the 21 autosomal microsatellite loci used in the wildcat there was no detectable signal in the fragment analyses. Three of the other 20 loci were monomorphic, and four further loci were dimorphic. These markers were not used in the presentation of the results. Eight of the field samples had the same genotype, whereas one sample had a difference at two loci and another sample at one locus. In sample 2474 no allele length was detected at locus F115. The differences in these samples were manifested as homozygous loci, which may also be allelic dropout due to

degradation of field scat and urine samples, thus field samples can be from the same individual. In all cases the samples were male for sexing. The number of alleles was 3-6, with an average allele number per locus of 3.69.

3.3. Wildcat

3.3.1. Genetic diversity

The MICRO-CHECKER software detected no PCR errors (scoring error, large allele dropout, false alleles) in the data set, but null alleles were detected at one locus in the group of domestic cats, and at three loci in the group of wildcats. These loci were not used in the presentation of the results, therefore I performed further analyses using a total of 17 loci. Diversity values per locus and per group can be found in Table 2.

Table 2: Diversity values of feline specific autosomal microsatellites (STR) in a group of domestic cat and wildcat.

	Domestic cat							Wildcat						
STR	N	N_A	H_O	H_E	HWE	PIC	I	N	N_A	H_O	H_E	HWE	PIC	I
FCA043	17	6	0.59	0.68	*	0.63	1.35	30	8	0.80	0.78	ns	0.75	1.69
FCA023	17	7	0.82	0.71	ns	0.67	1.50	30	8	0.80	0.72	ns	0.68	1.51
FCA097	17	9	0.65	0.80	*	0.78	1.84	30	8	0.83	0.77	ns	0.74	1.72
FCA132	17	11	0.82	0.88	*	0.87	2.23	30	11	0.80	0.76	ns	0.74	1.84
FCA223	17	9	0.76	0.81	ns	0.79	1.89	30	8	0.73	0.71	ns	0.67	1.52
FCA698	17	6	0.59	0.69	ns	0.66	1.45	30	9	0.73	0.73	*	0.68	1.59
FCA149	17	7	0.88	0.81	*	0.78	1.75	30	7	0.77	0.78	*	0.75	1.66
FCA310	17	6	0.47	0.53	ns	0.49	1.08	30	3	0.07	0.07	ns	0.06	0.17
FCA126	17	10	0.65	0.80	ns	0.78	1.91	30	7	0.83	0.77	ns	0.73	1.61
FCA220	17	3	0.47	0.38	ns	0.34	0.68	30	9	0.83	0.78	**	0.75	1.76
FCA090	17	6	0.71	0.65	ns	0.62	1.34	30	9	0.93	0.81	ns	0.79	1.83
FCA559	17	7	0.82	0.78	ns	0.75	1.70	30	4	0.47	0.47	ns	0.44	0.91
FCA008	17	9	0.82	0.77	ns	0.75	1.81	30	11	0.87	0.81	ns	0.79	1.96
FCA045	17	8	0.65	0.80	*	0.77	1.77	30	9	0.80	0.83	ns	0.8	1.91
FCA001	17	8	0.94	0.79	ns	0.76	1.76	30	9	0.93	0.81	***	0.79	1.85
FCA506	17	11	1.00	0.87	*	0.85	2.16	30	13	0.97	0.85	*	0.84	2.20
F115	17	12	0.82	0.83	ns	0.82	2.10	30	13	0.83	0.89	ns	0.88	2.30
Overall	17	7.94	0.73	0.74	-	0.71	1.67	30	8.59	0.76	0.73	-	0.70	1.65

Number of individuals (N), number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, Hardy-Weinberg equilibrium (HWE), Polymorphism Information Content (PIC), Shannon's Information Index (I). ns = non-significant; * = $p<0,05$; ** = $p<0,01$; *** = $p<0,001$.

3.3.2. Genetic structure and hybridisation

The software STRUCTURE detected the highest average log-likelihood values for two genetic clusters (K = 2). In this case, domestic cats (coloured green) clustered in one group and wildcats (coloured red) in another group. When examining individual genotypes within the clusters, the wildcat cluster

contains putative wildcat individuals, less than 75% ($q^{(i)} \leq 0.75$ assignment value) genetically belong the cluster, but more than 25 % ($q^{(i)} \geq 0.25$ assignment value) are genetically similar to the domestic cat cluster. I considered these samples as hybrids (Figure 3/c I, II, III). The wildcat cluster also includes a putative wildcat sample that is less than 25% ($q^{(i)} \leq 0.25$) genetically similar to the wildcat cluster, but more than 75% ($q^{(i)} \geq 0.75$) genetically similar to the domestic cat cluster (Table 3). This individual is a domestic cat based on the analyses.

Table 3: $q^{(i)}$ values from the STRUCTURE analysis.

Sample ID	$q^{(i)}$ value for domestic cat group	$q^{(i)}$ value for wildcat group
I.	0.64	0.35
II.	0.67	0.33
III.	0.52	0.48
IV.	0.84	0.16

For field wildcat samples I, II and III this value is greater than 0.25 and less than 0.75, which indicates that these individuals are hybrids. For individual IV, this value is greater than 0.75 in relation to the domestic cat group, and therefore this individual is considered to be a domestic cat.

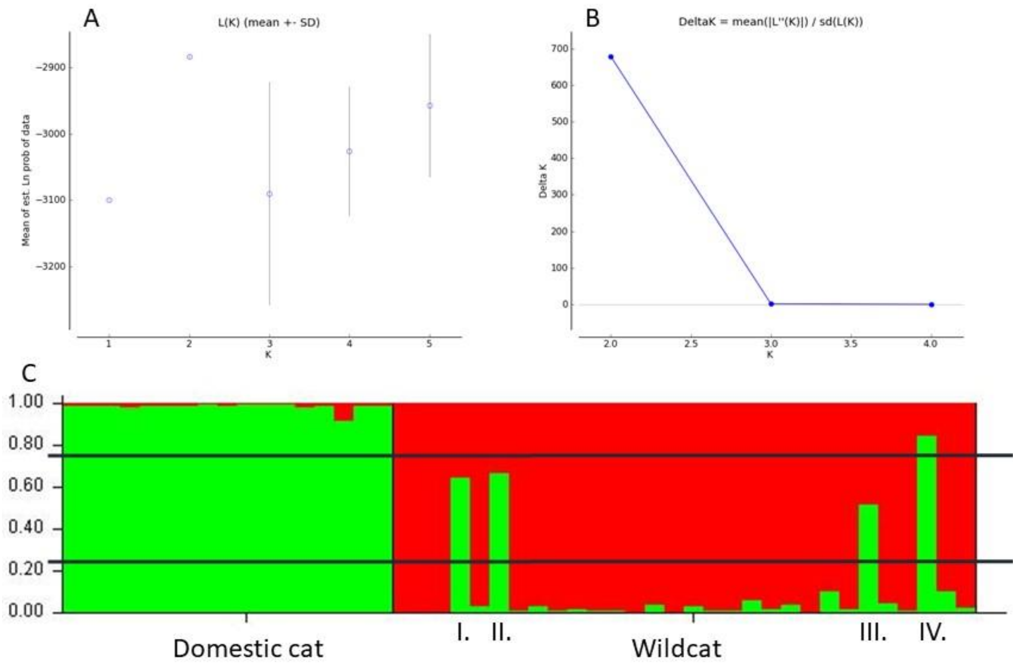

Figure 3: Log-likelihood values (L) („A") and their variation (DeltaK) for successive clusters („B") calculated from autosomal STR markers based on Structure analysis. Based on the highest DeltaK value, the decomposition into two clusters (K = 2) is the most probable, the result of which is shown in Figure „C".

Genotypes that were generated in Hybridlab software were tested by STRUCTURE software. Using an assignment value of $q^{(i)} \geq 0.75$, the generated domestic cat and wildcat genotypes were separated 100% (Figure 4). Among the generated F1 hybrids, 10% of the genotypes were incorrectly assigned to the domestic cat or wildcat group by the software based on $q^{(i)}$. In the case of the generated F2 hybrids, this value was 20% (Table 4).

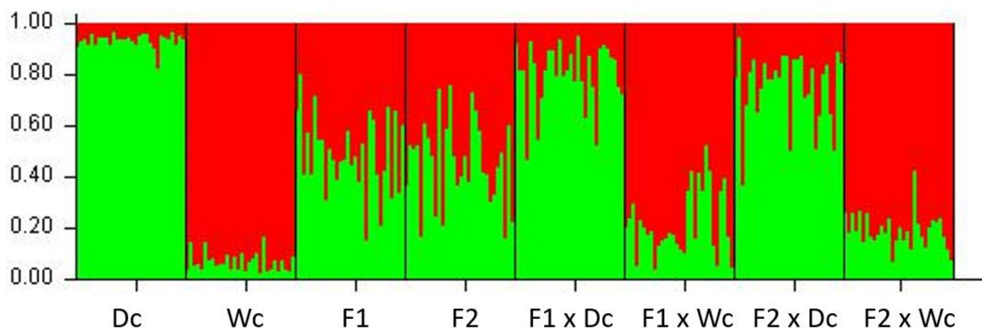

Figure 4: Structure analysis of parental and different hybrid groups generated with autosomal STR markers in Hybridlab. Thirty genotypes were generated in each group, with two clusters (K = 2) according to the highest DeltaK value. Dc – Domestic cat; Wc – wildcat; F1 – First hybrid generation; F2 – Second hybrid generation.

Table 4: Average $q^{(i)}$ values for the Structure analysis and the ranges of generated genotypes for the parental and different hybrid categories.

Artificially generated genotype groups	Average $q^{(i)}$ value (range)	Unclassified ($q^{(i)} < 0.75$; %)
Domestic cat	0.94 (0.83 – 0.97)	0
Wildcat	0,93 (0.83 – 0.97)	0
F1	0.51 (0.2 - 0.85)	90
F2	0.54 (0.24 - 0.84)	80
F1 X Dc	0.80 (0.47 - 0.95)	26.67
F1 X Wc	0.78 (0.48 - 0.96)	30
F2 X Dc	0.76 (0.37 - 0.95)	36.67
F2 X Wc	0.81 (0.58 - 0.93)	16.67

Column 3 shows the % of genotypes not belonging to any parental group. Values above 0.75 for $q^{(i)}$ indicate membership of a parental group.

4. DISCUSSION AND RECOMMENDATION

4.1. Grey wolf

The canine microsatellite markers we used were suitable for the grey wolf and showed a sufficient number of polymorphisms to assess genetic diversity and genetic structure. We found moderate levels of genetic diversity in Hungarian wolves ($H_o = 0.60$; $uH_E = 0.69$). Similar levels of heterozygosity were found in Slovakia by Rigg et al. (2014) ($H_o = 0.65$, $H_E = 0.64$), Szewczyk et al. (2019) ($H_o = 0.65$, $uH_E = 0.678$) and Hulva et al. (2018) ($H_o = 0.694$, $H_E = 0.733$) and in Serbia, including the southern-most portion of the Carpathians, by Đan et al. (2016) ($H_o = 0.69$; $H_E = 0.75$). Bakan et al. (2014) also reported heterozygosity of Slovakian ($H_o = 0.539$; $H_E = 0.707$) and Serbian ($H_o = 0.526$; $H_E = 0.637$) samples. These findings are consistent with studies of the species elsewhere in Europe (Hindrikson et al. 2017) except in Italy, where heterozygosity was found to be lower ($H_o = 0.57$; $uH_E = 0.58$) as a result of the population passing through a severe genetic bottleneck (Fabbri et al. 2014). The results of our analyses of the genetic structure suggest that the Slovakian population probably contributed to the gene pool of Hungarian wolves, likely via natural dispersal, but it may not be the only population involved in the recolonisation of Hungary. Wolves are capable of dispersing over long distances (e.g. Wabakken et al. 2007; Ciucci et al. 2009; Andersen et al. 2015, Bartoń et al. 2019, or the latest result from Switzerland). Our results do not support the hypothesis that the presence of free-ranging wolves in northern Hungary is the result of releases from zoos or other captive facilities (cf. Kovács 2018; Fluck 2020). However, the number of samples from captive wolves used was limited, and although we cannot completely exclude the possibility of such releases, this seems unlikely based on the observed genetic structure. The dispersion may be facilitated by suitable habitats and ecological

corridors (Köck et al. 2014), so animals originating from different populations can mix and contribute to the present genetic pool of a given population, as has been proved in several regions in Europe (e.g. Ražen et al. 2016; Hulva et al. 2018; Szewczyk et al. 2019). Thus, Hungarian wolves separating from the Slovakian population and captive individuals at K = 4 in the admixture analyses may also be immigrants from other populations or their descendants. Wolves can disperse into Hungary via least-cost paths to core areas in the Börzsöny, Bükk, Mátra and Zemplén Mountains, where most of the samples for this study were collected.

Our Hungarian wolf samples were slightly male-biased, which might reflect differences in dispersal between the sexes. The dispersal of natural grey wolf populations is usually male-biased, i.e. males seem to have a greater tendency to disperse (Stansbury et al. 2016). This suggests that the wolf population in Hungary is still in the initial reintroduction phase and still heavily dependent on replenishment from Slovakian source populations.

4.2. Eurasian lynx

A very low number of alleles per locus was observed in reference samples from Romania and Slovakia as well as among the field samples. Most of the studies on the genetics of the Eurasian lynx use microsatellite loci developed from the domestic cat genome (Menotti-Raymond & O'Brien 1995, Menotti-Raymond et al. 1997, Menotti-Raymond et al. 1999). In addition to these loci, others originally developed for the Canadian lynx (*Lynx canadensis*) and the Sumatran tiger (*Panthera tigris sumatrae*) are also used for microsatellite-based studies of the Eurasian lynx (Carmichael et al. 2002, Williamson et al. 2002). One reason for the low genetic diversity in a large part of European populations is low founder numbers in artificial reintroductions (Linnell et al. 2009). Microsatellite-based analyses of lynx samples collected over several

years in the Börzsöny mountains identify a single individual, which confirms the camera trap results of field specialists, suggesting that there is probably one individual in the mountain range (Péter Bedő, László Darányi verbal communication). Our results, obtained from reference samples from Slovakia and Romania and captive samples, furthermore the male individual from field samples, showing a low number of alleles per locus are in agreement with reports of low level of heterozygosity in lynx in the Carpathians (Schmidt et al. 2011, Krojerová-Prokešová et al. 2019).

4.3. Wildcat

Most of the microsatellite-based studies in the European wildcat population have been performed using loci described in domestic cats by Menotti-Raymond & O'Brien (1995) and Menotti-Raymond et al. (1999, 2003) (pl. Randi et al. 2001, Lecis et al. 2006, Hertwig et al. 2009, Eckert et al. 2010, Say et al. 2012, Steyer et al. 2013, Mattucci et al. 2013, Mattucci et al. 2016).

For the Hungarian samples the observed heterozigosity (H_O) was 0.76 and the expected heterozigosity (H_E) was 0.73. Similar levels of heterozygosity were found in France by Say et al. (2012) (H_O = 0,70; H_E = 0,73), and in Croatia by Urzi et al. (2021) (H_O = 0,72; H_E = 0,72). Identification of hybrid individuals beyond the first generation (F1) is usually characterized by weak discriminatory power, as it has been shown using Bayesian approaches for simulated data of microsatellites (Vähä & Primmer 2006), or by simulating hybrid individuals using empirical microsatellite data from wildcat and domestic cat parents (Hertwig et al. 2009, O'Brien et al. 2009, Oliveira et al. 2008). My results based on simulated genotypes show that the microsatellite loci I used are highly (90%) suitable for separating the parental and admixture individuals up to the first hybrid generation (F1). However, microsatellites did

not allow to distinguish different hybrid classes, including backcrosses. Steyer et al. (2018) also performed an SNP-based analysis, and found that the microsatellites they used were 100% suitable for separating the first hybrid generation (F1); however, the loci they used were not suitable for separating the other hybrid classes. On the other hand, the SNP genotypes they simulated were capable of separating a large portion of the second hybrid generation (F2) from the parental generations (12% error between simulated genotypes). However, the success in identifying simulated hybrids is limited beyond the second generation, in agreement with Nussberger et al. (2013).

4.4. Recommendation

Genetic methods can provide important information on the past and present status of populations, complementing field data collection methods, especially in the case of cryptic species, where monitoring their status is difficult. As these populations are often located in several countries and have been shown to migrate long distances, it would be important to use a standardised genetic methodology to enable comparison of the results across Europe and, in particular, in the Carpathian region. This would greatly facilitate monitoring population status and potential further expansion, and would contribute to transboundary conservation and management programmes following European Union guidelines.

Our analyses revealed that the Slovakian population probably contributed to the gene pool of Hungarian wolves via natural dispersal. However, to investigate if Slovakia is the source of the recolonization of Hungary or whether other wolf populations have also contributed, reference samples from additional regional populations need to be included in future analyses. In addition, it would be advisable to follow a standardized sample collection protocol and investigational methodology in Hungary, in order to obtain more

accurate results on the current status of the large carnivore population in the country. I studied the genetic diversity and origin of the grey wolf in Hungary using 14 microsatellite loci and a marker for sex determination. These markers are suitable for species and individual identification, as well as for the assessment of kinship relations. By increasing the number of Eurasian lynx samples collected in the Carpathian Basin and Hungary and using more microsatellite loci, more detailed data could be obtained on the current status of the species. Hybridisation has already been detected in wildcats in Hungary in the early 2000s. However, in order to obtain more accurate results on the genetic status and level of hybridization of the Hungarian wildcat population, it would be necessary to collect samples from known occurrences of the species. More accurate determination of hybridization levels would necessitate the application of Single Nucleotide Polymorphism (SNP) panels, as data in the literature suggest that certain SNP panels are able to identify hybrid individuals with higher genetic probability, even in the second hybrid generation (F2).

5. NEW SCIENTIFIC ACHIEVEMENTS

1. I have provided evidence that the grey wolf has been reintroduced to the North Hungarian Mountains from the Slovakian population through natural dispersal. Furthermore, my analysis does not support the hypothesis that individuals from zoos have been involved in the reintroduction of the Hungarian grey wolf population.
2. Based on genetic analysis of field-collected grey wolf samples, I estimated the minimum number and sex ratio. I found that several individuals at different times have been involved in the reintroduction of the Hungarian grey wolf population.
3. The reconstructed kinship relationships confirm that the wolf population studied in the North Hungarian Mountains is not just in the course of arriving to their new location, but already has established breeding pairs.
4. I have successfully carried out the first genetic-based occurrence study on the Eurasian lynx in Hungary, mostly on samples from the Börzsöny Mountains. I proved that the samples collected over several years using non-invasive methods were from a single male individual.
5. I have assessed the genetic diversity of a large population of wildacts in the North Hungarian Mountains. Using microsatellite markers adapted and optimized from the international literature, I successfully classified the samples into European wildcat, domestic cat and hybrid groups. I also examined the reliability of these markers for hybridisation, and found that first-generation hybrid individuals (F1) can be separated with 90% confidence using these markers.

6. SCIENTIFIC PUBLICATIONS

Articles published in journals with impact factors, related to the topic of the thesis:

❖ **Fehér P.***, Frank K.*, Gombkötő P., Rigg R., Bedő P., Újváry D., Stéger V.✉, Szemethy L.✉ (2022): The origin and population genetics of wolves in the north Hungarian mountains. Mammalian Biology, open access. https://doi.org/10.1007/s42991-022-00287-7: **Q1** IF: **1,99** független idéző közlemények száma: **3**

❖ Kemenszky P.*, **Fehér P.***, Farkas A., Jánoska F., Frank K., Bedő P., Barta E., Varga L., Szemethy L.✉, Stéger V.✉ (2021): Genetic differentiation of the Golden Jackal (Canis aureus) populations in southern Hungary and southern Romania as revealed by microsatellite data analysis. North-Western Journal of Zoology, 17 (1), 111-116. **Q3** IF: **0,78** független idéző közlemények száma: **1**

❖ Schally G., Frank K., Heltai B., **Fehér P.**, Farkas Á., Szemethy L., Stéger V. (2018): High genetic diversity and weak population structuring in the Eurasian Woodcock in Hungary during spring. Ornis Fennica, 95 (2), 61-69. **Q2** IF: **1,39** független idéző közlemények száma: **3**

Articles published in journals without impact factors, related to the topic of the thesis:

❖ **Fehér P.**, Frank K., Szemethy L., Stéger V. (2020): Nagyaragadozók Magyarországon II., Molekuláris biológiai módszerek a vadbiológiában. WWF Magyarország Alapítvány, Budapest. 23pp.

Educational articles related to the topic of the thesis:

❖ Frank K., **Fehér P.**, Mihalik B., Stéger V. (2019): Genetikai vizsgálatok a vadgazdálkodás szolgálatában. Vadászévkönyv 2019, Dénes Natúr Műhely, 150-157p.

Oral presentations related to the topic of the thesis:

❖ Gombkötő P., **Fehér P.**: Vadmacska GSM alapú egyedi jelölésének első tapasztalatai a Bükki Nemzeti Park Igazgatóság működési területén. A lopakodás nagymestere végveszélyben II., A vadmacska (Felis silvestris) helyzete a Pilis-Budai hegység térségében szakmai konferencia, Budakeszi Vadaspark, 2022.12.08. – meghívott előadó

❖ **Fehér P.**, Gombkötő P., Bedő P., Frank K., Ninausz N., Szemethy L., Stéger V. (2021): A szürke farkas (Canis lupus) visszatelepülésének nyomon követése molekuláris genetikai módszerek segítségével. Emlőskutatók Szakmai Napja 2021, Budapest, 2021.12.09., 9-10 p., ISBN: 9789639877467

❖ **Fehér P.**: Molekuláris genetikai módszerek alkalmazása a hazai vadmacska monitoringban. A lopakodás nagymestere végveszélyben I., A vadmacska (Felis silvestris) helyzete a Pilis-Budai hegység térségében szakmai konferencia, Budakeszi Vadaspark, 2021.11.03. – meghívott előadó

❖ **Fehér P.**: Genetikai módszerek alkalmazása a hazai ragadozó fajok monitorozásában. Life EuroLargeCarnivores Program, Haszonállat védelem- és nagyragadozók szakmai konferencia, Parádfürdő, 2021.10.20-22. – meghívott előadó (magyar nyelvű)

❖ **Fehér P.**: Genetika a természetvédelem és a vadgazdálkodás szolgálatában. Life EuroLargeCarnivores Program, Falka Napok, Aggtelek, 2021.08.07-08. – meghívott előadó (magyar nyelvű)

- ❖ **Fehér P.**, Ninausz N., Pásztor A., Szabó L., Szemethy L., Heltai M., Stéger V., Varga L. (2021): Analysis of MC1R pigmentation gene in the Hungarian population of golden jackal (Canis aureus). 18th Wellmann International Scientific Conference, 2021.05.13., 31 p., ISBN: 9789633067901
- ❖ **Fehér P.**, Gombkötő P., Bedő P., Rigg R., Frank K., Varga L., Barta E., Szemethy L., Stéger V.: Molekuláris biológiai módszerek a vadbiológiában. Life EuroLargeCarnivores Program, Nagyragadozók jelenlétének kimutatása online workshop. 2020.12.16. – meghívott előadó (magyar nyerlvű)
- ❖ **Fehér P.**, Frank K., Szepesi K., Heltai B., Barta E., Újváry D., Gobkötő P., Szemethy L., Stéger V. (2017): A hazai macskafélék (Felidae) genetikai monitorozási módszerének fejlesztése. Magyar Biológiai Társaság XXX. Vándorgyűlése, Budapest, 2017.02.17-18., 45-46 p. ISBN:9789638734389.

Poster presentations related to the topic of the thesis:

- ❖ **Fehér P.**, Frank K., Kemenszky P., Farkas A., Jánoska F., Bedő P., Barta E., Varga L., Szemethy L., Stéger V. (2022): A population genetics-based study of the rocolonization of the golden jackal (Canis aureus) in two core areas in southern Hungary and southern Romania. 3rd International Jackal Symposium, Gödöllő, 2022.11.02-04., 59 p., ISBN: 9789636230128
- ❖ **Fehér P.**, Heltai B., Frank K., Kemenszky P., Jánoska F., Szemethy L., Varga L., Stéger V. (2019): Population genetic survey of the golden jackal (Canis aureus) in the southern transdanubian region. 17th Wellmann International Scientific Conference, Hódmezővásárhely, 2019.05.08., 26-27 p., ISBN: 9789633066539

- **Fehér P.**, Szepesi K., Frank K., Heltai B., Mihalik B., Újváry D., Szilágyi I., Gombkötő P., Szemethy L., Stéger V. (2018): Development of genetic monitoring methods for Hungarian large carnivores. Fiatal Biotechnológusok Országos Konferenciája (FIBOK 2018), Budapest, 2018.03.28-29., 67 p., ISBN: 9789633153703
- **Fehér P.**, Frank K., Heltai B., Szepesi K., Mihalik B., Barta E., Újváry D., Gombkötő P., Szemethy L., Stéger V. (2017): Genetic monitoring of Hungarian carnivores. Hungarian Molecular Life Sciences 2017, Eger, 2017.03.31-2017.04.02., 249-250 p. ISBN: 9786155270345

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