

# WILDLIFE BIOLOGY

## Short communication

## Genetic admixture between Central European and Alpine wolf populations

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The recovery and expansion of formerly isolated wolf populations in Europe raise questions about the nature of their interactions and future consequences for population viability and conservation. Will fragmented populations fuse or maintain a certain level of isolation with migration? Central Europe is suitable for obtaining empirical data in this field as it represents a 'crossroad' with the potential for contact among several phylogeographic lineages. In this study, non-invasive genetic samples obtained during population monitoring in the Bohemian and Bavarian Forest (BBF) mountain ranges in the Czech Republic and Germany (Bohemian Massif) were analysed at different neutral markers including mitochondrial sequence, nuclear autosomal microsatellites and gonosomal sex markers. Resultant genetic profiles were compared with reference data to study population ancestry. Both cluster analyses of microsatellite genotypes and syntopic occurrence of haplotypes HW01 and HW22 showed genetic admixture between Central European and Alpine populations. This represents secondary contact and interbreeding of formerly allopatric populations with different phylogeographic histories and distant expansion centres in different biomes in the Baltic region versus the Apennine peninsula and Alps. Moreover, the study describes the founding event and genealogy of this admixed deme, inhabiting intermediate environmental conditions compared to parental forms, and emphasises the role of protected areas as stepping stones in the range recolonization process in endangered large mammals.

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## Introduction

Range history of many megafaunal species frequently involves 1) large continuous ranges prior to substantial human impacts; 2) anthropogenic range reduction and fragmentation; 3) in some cases, as in the grey wolf *Canis lupus*, a re-expansion process in the post-industrial era. Various studies highlight that European mammal populations were more interconnected before human interventions (Tucker et al. 2018). Range fragmentation as a result of habitat loss and exploitation/persecution has often profound consequences on genetic variation and structure in particular species (Haag et al. 2010). Protection measures, landscape abandonment and other factors lead to the recovery of some populations (Horn et al. 2014, Lucena-Perez et al. 2022). However, describing consequences and predicting the future of the recolonization and expansion process in formerly isolated animal populations is challenging. One possible scenario involves the fusion of populations, erasing genetic differences (Kearns et al. 2018, Jan et al. 2023). On the other hand, a certain level of isolation with migration maintained by ecological differentiation could occur in species with ranges spanning across different biomes, including cosmopolite predators (Pilot et al. 2006, Ratkiewicz et al. 2014, Foote et al. 2016).

A crucial proximate factor involved in expansion scenarios is the intraspecific genetic admixture of formerly isolated divergent lineages. Admixture could have negative consequences, such as outbreeding depression (Sagvik et al. 2005, Huff et al. 2011). Specific conditions have to be met if the process has to involve beneficial effects related to heterosis and play an adaptive role. During range expansions, intraspecific admixture might generate changes in genomic compositions that can eventually increase fitness and provide opportunities for local adaptation and survival (Krehenwinkel and Tautz 2013, Rius and Darling 2014, Fontseré et al. 2019). For example, Hendricks et al. (2019) reported admixture between coastal rainforest and Northern Rocky Mountain forest wolf ecotypes. Admixed packs colonised new environmentally intermediate areas in the state of Washington, USA. Notably, one admixed pack even created a territory in an area where the environment was less suitable for both parental populations (Hendricks et al. 2019). Recent beneficial admixture events among several divergent phylogeographic lineages, formed during dramatic local depletions caused by hunting and habitat destruction, were reported in several European mammal species. For instance, the Eurasian otter *Lutra lutra* (Pigneur et al. 2019), the European beaver *Castor fiber* (Munclinger et al. 2022), the European wildcat *Felis silvestris* (Mueller et al. 2020) or brown bear *Ursus arctos* (Kopatz et al. 2014).

Central Europe represents an important phylogeographic 'crossroad' where various previously isolated populations interact (Eliášová et al. 2022). The wolf populations provide a

compelling example of the interactions and genetic divergence shaping the region's biodiversity (Pilot et al. 2010). Currently, Central European, Carpathian, Alpine and Dinaric-Balkan populations occur within the area (Hulva et al. 2018).

The Central European population diverged from the Baltic population (Szewczyk et al. 2019, 2021). Following the nationwide protection of wolves in Poland in 1998, the founders of this population expanded from northeastern Poland toward the west (Nowak and Mysłajek 2016) and into Germany (Jarausch et al. 2021), where they established the first breeding pack in 2000 (Ansorge et al. 2010). The current range of Central European population is situated in parts of the European plain, with the largest numbers in Poland and Germany and offshoots into other areas such as the Czech Republic (Fig. 1, Supporting information, Boitani 2018) and isolated enclaves in Slovakia (Hulva et al. 2018). Therefore, also some degree of admixture between Central European and Carpathian populations was referred to (Hulva et al. 2018).

The Alpine population originates from an isolated Italian population in the Apennine Peninsula and colonised the Alps during the 1990s (Lucchini et al. 2002, Valière et al. 2003). The colonisation of the Alps involved a strong founder effect, and as a result, individuals forming the present population in the Alps show lower genetic variability compared to Apennine wolves (Fabbri et al. 2007). Genetic differentiation is maintained by reduced gene flow between the source population in Italy and the population in the Alps (Fabbri et al. 2007, 2014). However, some admixture of the Alpine population with the Dinaric-Balkan population was detected (Ražen et al. 2016), and presumably, it could be related to the colonisation of new areas (Fabbri et al. 2014). Currently, the majority of the population inhabits the western parts of the Alps, between France and Italy, with a smaller proportion of the population inhabiting the eastern part of the Alps (Marucco et al. 2023). Several territories are also established in Massif Central, Pyrenees, Switzerland, and Austria (Boitani 2018). Vagrant animals have also been occasionally documented in Germany and the Czech Republic (own data).

The investigated populations exhibit contrasting phylogeographic histories (Pilot et al. 2010, Hindrikson et al. 2017, Montana et al. 2017, Salari et al. 2017). Wolves from the Italian and Alpine populations are characterised by a unique haplotype belonging to haplogroup 2 and are related to an ancient extinct lineage that was widespread in Eurasia and North America during the late Pleistocene (Pilot et al. 2010), including a distinct morphotype of Beringian wolves (Meachen et al. 2016). Wolves from the Central European population, in contrast, belong to haplogroup 1, which increased in frequency after the last glacial maximum (LGM) and outnumbered haplogroup 2 in the last several thousand years (Pilot et al. 2010, Hindrikson et al. 2017). Changes in haplotype frequencies were most likely associated with environmental and ecological changes occurring after the LGM

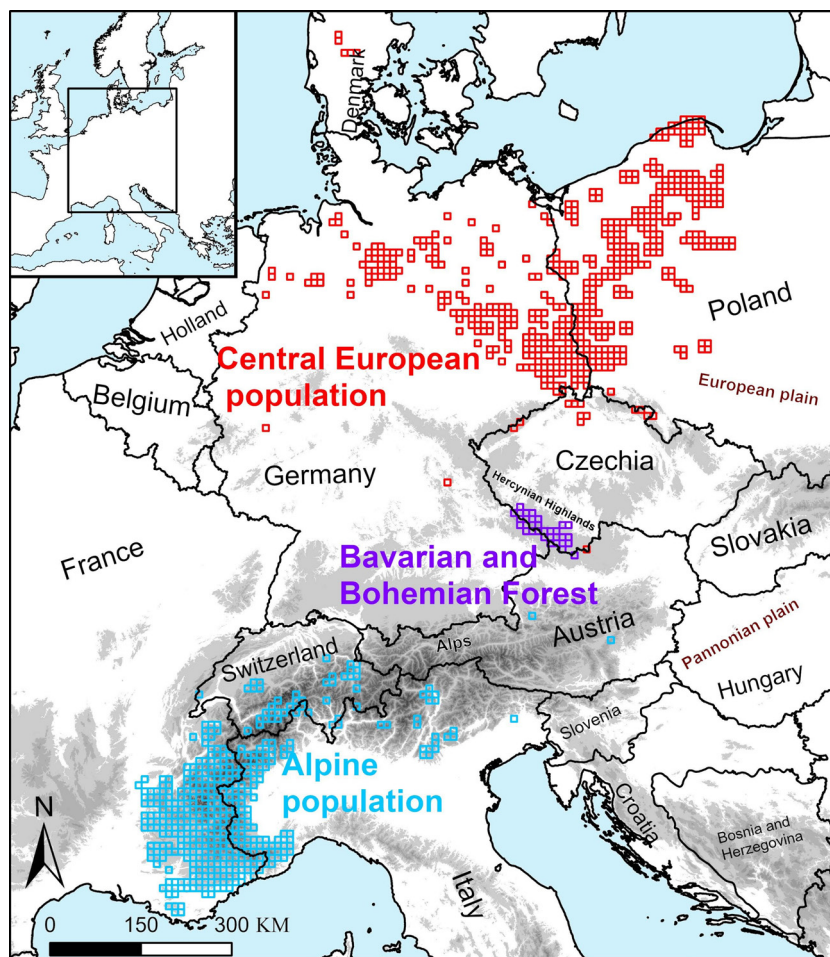


Figure 1. Permanent occurrence of grey wolves from cores of Alpine (blue) and Central European (red) populations, together with all occurrence data from the Bohemian and Bavarian Forest (dark purple). Occurrence data for Central European and Alpine populations contain data from the IUCN red list (data until 2016) updated with more recent publications and available reports (for some countries, up until the wolf year 2019–2020). Data for the Bohemian and Bavarian Forest contain all presence data from the years 2014–2021 within the map area range. A list of data sources is found in the Supporting information. Occurrence data are shown in the EEA reference grid (10 × 10 km). Altitude is displayed in grey tones scaled by 500 m with an altitude range of 0–4800 m.

(Pilot et al. 2010). Divergent microevolutionary pathways are also mirrored in phenotypic diversification. Following Bergmann's rule, wolves from the Italian and Alpine populations have smaller body sizes than Central European wolves (Nowak and Federoff 2002, O'Keefe et al. 2013). These populations also differ in phenotypic traits and behaviour, related to variations in hunting strategies and prey preferences (Palmegiani et al. 2013, Zlatanova et al. 2014), and reproduction dynamics, particularly the timing of reproduction (Mech 2002, Schmidt et al. 2007). Denning behaviour also varies; lowland females dig dens under uprooted trees etc., while mountain females use natural hiding places (caves and rock crevices) as dens (Mech and Boitani 2003, Schmidt et al. 2007, Volokh 2011).

This study is focused on the recolonization of the Bohemian and Bavarian Forest (BBF) Ecosystem by grey wolves using non-invasive genetics. First, it examines its ancestry within Central European and Alpine populations and the degree of admixture within the BBF deme using

clustering methods. Second, based on genealogical methods, it describes its relationships with parental populations originating in the Alps and North European Plain, its genetic composition and pack history.

## Material and methods

### Study area

The Bohemian and Bavarian Forest (BBF) is located in a geological unit that forms the transition from the Great European Plain in the north to the Hercynian orogenic belt in the south (Fig. 1), typical of higher altitudes. BBF includes one of the largest protected contiguous forests in Central Europe (Heurich et al. 2015). It is dominated by forested low mountains, with peatlands in certain areas, and vegetation closely resembling alpine high mountain flora (Metzger et al. 2005). The local wolf population became extinct in the 18th century (Hürka 1981, Bufka et al. 2005).

In 1963, nature protection efforts led to the creation of the Šumava protected landscape, which covers approximately 996 km<sup>2</sup>. The Bavarian Forest (Bayerische Wald) National Park was founded in 1970 on an area of 250 km<sup>2</sup>, and the Šumava National Park (Bohemian Forest National Park) was established in 1991, with an area of approximately 690 km<sup>2</sup> (Křenová and Vrba 2014). The presence of wolves was sporadic until the first evidence of the permanent settlement of wolves in this area in 2015 (Kutal 2017).

### Field monitoring and sample collection

Genetic and occurrence data were collected in the BBF during the wolf years (WY) (start of May to the end of April the following year) from WY 2013/2014 to WY 2020/2021 (Supporting information). Whilst the dataset contains data up until 7 March 2021, the entire WY 2021/2022 could not be considered and evaluated in this study. Before 2017, wolves were monitored opportunistically in the study area; whereafter systematic monitoring was applied. For the genetic analysis, non-invasive samples of scats, hairs, urine, urine swabs, female oestrus blood, swab samples from kill sites, and tissues from roadkill were included (Supporting information). Samples were collected by the responsible organisations (c.f. Acknowledgements). All samples were stored in 96% ethanol at −20°C. Occurrence data consisted of camera-trapping and track data, following the approach of Kaczensky et al. (2009). Each record was positioned by a handheld GPS and dated. The camera traps were placed on sites where signs of wolf presence had been previously evidenced.

All records were validated according to the ‘status and conservation of the alpine lynx population’ (SCALP) criteria (Molinari-Jobin et al. 2012). For the assessment of the SCALP value for wolves, the guidelines published by Kaczensky et al. (2009) were used. For the spatial visualisation, only records that approved the C1 (clear evidence: e.g. telemetry data, genetic evidence, animal found dead, camera trap pictures) or C2 (evidence confirmed by an experienced person: e.g. track or prey) validation were considered; all such records were converted to the point layer in the geographical information system (GIS) (ArcGIS 10.8 by ESRI). Thus, each given point represents one confirmed wolf’s mark for the date of origin. The spatial context of the occurrence data was investigated in ArcGIS 10.8 by ESRI. The records were grouped into wolf years (WY). The Kernel Density Estimation was deployed to show hot spots of wolf activity in the last complete WY available for our study – WY 2020/2021. The kernel function used the quartic kernel function (Silverman 1998); the bandwidth was calibrated using the data from the given WY. Areas covered by successfully genotyped samples of two wolf packs were visualised as 100% minimum convex polygon (MCP) based on genetic samples from WY 2020/2021, presumably after wolf territory stabilisation. The area of the polygons was measured in ArcGIS 10.8 by ESRI.

Information on the permanent occurrence of Alpine and Central European populations (Fig. 1), together with occurrence data of other populations located in the distance from

the BBF reachable by wolf dispersal were taken from the available literature until the year 2020 (Supporting information) and merged into the occurrence layer in ArcGIS 10.8 by ESRI. Reference genetic data used for comparative cluster analyses included samples from Alpine and Central European populations (Supporting information). Genotypes from the enclosures in the Bohemian and Bavarian Forest were added to detect the potential contribution of animals from captivity in the wild population (Supporting information).

### DNA extraction and amplification

DNA from faecal samples was extracted using a silica-binding extraction kit (QIAamp Fast DNA Stool Mini Kit, Creel et al. 2003). The final step of the protocol was modified, as DNA was eluted with 100 µl of elution buffer instead of 200 µl. DNA from hair/swab/muscle tissue samples was isolated using the tissue genomic DNA mini kit (GENEAID) according to the manufacturer’s protocol. DNA isolation from urine samples followed the methodology of Hausknecht et al. (2007) with minor modifications (Jarusch et al. 2021), together with the protocol of the commercial kit QIAamp Fast DNA stool mini kit. DNA from the oestrus blood samples was extracted using the commercial blood/cell DNA mini kit (GENEAID).

A combination of parentally inherited autosomal microsatellites, gonosomal sex-determining loci, and matrilineal mitochondrial DNA were used for individual genotyping and haplotype determination. A total of 21 microsatellite loci were used, of which 13 loci were shared by the Czech and German sides (Supporting information): FH2001, FH2010, FH2017, FH2087, FH2088, FH2096, FH2137, FH2140, FH2054, FH2161 (Francisco et al. 1996), vWF (Shibuya et al. 1994), CPH5 (Fredholm and Winterø 1995) and PEZ17 (Neff et al. 1999). Samples processed on the Czech side were supplemented with loci INRA21 (Mariat et al. 1996), FH2097 (Francisco et al. 1996), CXX279 (Ostrander et al. 1993), REN169O18, REN169D01 (Guyon et al. 2003), AHTk211 (Lingaaas et al. 1997), REN64E19 (Breen et al. 2001), and INU055 along with a sex determination gene (amelogenin; Randi et al. 2014). Sex determination in samples obtained from Germany was determined using the DBX6 and DBY7 loci, according to Jarusch et al. (2021). All nuclear loci were amplified using fluorescently labelled primers. A multiplex approach (Taberlet 1996) comprising two to five replicates for each sample was applied. To minimise genotyping errors, a minimum of three independent PCR repeats were required for the homozygous locus (Tumendemberel et al. 2019). The PCR was set according to the manufacturer’s protocol for the multiplex PCR plus kit (QIAGEN) with an annealing temperature of 60°C. Amplicons for fragment analysis were mixed in a solution containing formamide and GeneScan™ 500 LIZ™ dye Size Standard (Thermo Fisher Scientific) and run on the Genetic Analyzer 3130xl (Applied Biosystems) with the standard DS-33. To amplify the left hypervariable domain of the mtDNA control region (Pilot et al. 2010), the methods of Hulva et al. (2018) and

Jarausch et al. (2021) were used. The study was enabled due to the activities of the CEwolf consortium, which aims at a harmonised transboundary genetic monitoring of the Central European wolf population (Szewczyk et al. 2021).

## Population genetics analyses

Allele scoring and binning were carried out in geneious software using the geneious Microsatellite Plugin (Kearse et al. 2012). Alleles at a particular locus were accepted according to the 'n/2' consensus method, in which the alleles will appear in half of the replicates (Benschop et al. 2013). In this study, the alleles that occurred in at least two out of three amplifications were admitted in the consensus genotype. Based on assumption of  $PI_{\text{sib}} < 0.001$  to ensure sufficient statistical power of dataset in identity and parental analyses (McKelvey and Schwartz 2004), samples with at least 67% loci amplification (minimum 14 out of 21 loci) and 77% loci amplification (10 out of 13 loci) were considered as multilocus consensus genotypes of sufficient quality in the Czech Republic and Germany, respectively (Szewczyk et al. 2019, Jarausch et al. 2021). Obtained sequences were edited, aligned, and assigned to the known haplotypes sensu Pilot et al. (2010) using geneious software (Kearse et al. 2012). Hardy–Weinberg equilibrium, presence of null alleles and allele dropout were estimated in a micro-checker (Van Oosterhout et al. 2004).

The identity analysis was performed using the cervus program (Kalinowski et al. 2007). For initial filtering, the minimum number of matching loci and fuzzy matching (number of mismatches between genotypes) within the Czech samples were set up to 14 and five loci, respectively. The comparison of Czech and German samples was based on 13 loci ( $P_{(\text{ID})\text{sib}} = 0.0002$ ) with the minimum number of matching loci being nine and five fuzzy matches, respectively. Before removing the identical genotypes from the dataset, primary data and metadata were examined again. The origins of the founding individuals and dispersal of the offspring outside of the studied area were determined through the comparison with genetic profile databases from national genetic wolf monitoring in Germany and the Czech Republic. ml-relate software (Kalinowski et al. 2006) was used to estimate the genealogical structure. After estimation of the most probable relationship (FS = full sibling; PO = parent/offspring), the specific hypothesis test, with 100 000 simulated genotypes, was run to determine if the chosen relationship was statistically significant (Kalinowski et al. 2006). Temporal and spatial data of the collected samples were considered to refine suggested relationships. Results from previous analyses were confirmed using a maximum-likelihood approach in colony ver. 2.0.6.6. (Jones and Wang 2010). The analysis was processed with the assumption of female/male polygamy and all individuals were considered as candidate parents. The probability of including fathers and mothers as candidates for parental pair was set to 0.5, with an assumed error rate of 0.01 (Palomares et al. 2017).

Bayesian clustering analyses were used to infer the population genetic structure. In the structure software (Pritchard et al.

2000), the initial burn-in was set to  $2 \times 10^5$  steps followed by  $10^6$  Markov Chain Monte Carlo (MCMC) iterations. The admixture model with correlated allele frequencies and no prior population information was used. The number of examined clusters was fixed from  $K=1$  to  $K=10$ . Each  $K$  was repeated five times. The results were uploaded to a web-based application structureselector (Li and Liu 2018) to select and visualise the optimal number of clusters using multiple methods. In this study, two methods were selected: the Puechmaille method (Puechmaille 2016) and the Evanno method (Evanno et al. 2005). Three different analyses in structure were performed. The first included all BBF individuals from the wild ( $n=22$ ) and captive individuals from the enclosures in the Bohemian ( $n=15$ ) and Bavarian Forest ( $n=8$ ). The second analysis contained only wild BBF individuals ( $n=22$ ). The third analysis was performed using only three individuals (offspring) per each pack from the wild BBF deme ( $n=6$ ), to control the impact of relatedness on the Bayesian clustering. In all three analyses, reference samples of the Alpine population ( $n=25$ ) and the Central European population ( $n=20$ ) were included. A factorial correspondence analysis (FCA) implemented in genetix 4.05 (Belkhir et al. 2000) was used to visualise genetic distances among the studied individuals and populations ( $n=67$ ). All unique genotypes from the BBF deme ( $n=22$ ) and comparison genotypes from the Alpine ( $n=25$ ) and Central European ( $n=20$ ) populations were used.

## Results

### Genealogy of BBF deme

A total of 233 genetic samples were processed in the study area during the period of interest. Totally 96 genotypes of wolves from the BBF were obtained and the amplification success rate was approx. 41% (Supporting information). The  $PI_{(\text{ID})\text{sib}}$  of the dataset including Czech and German samples (0.0002) was sufficiently low for the reliable discrimination of individuals (McKelvey and Schwartz 2004). The BBF deme is probably in HW equilibrium, no evidence of a large allele dropout was found, and the presence of null alleles was not detected. The identity analysis revealed 22 unique genotypes, yielding a recapture rate of 4.32. Eight females and 14 males were detected, resulting in a sex ratio of 1.75. In total, 18 mitochondrial sequences were identified from 22 samples with unique microsatellite genotypes (with a success rate of 82%; Fig. 2a). 17 individuals bearing the haplotype HW01 and one individual bearing the haplotype HW22 were identified in the study area. Captive individuals from enclosures bear different haplotypes than wolves from the BBF deme, captive wolves from the Bohemian Forest haplotype HW04 and captive wolves from the Bavarian Forest haplotype HW13 (Supporting information).

### Population structure and admixture analysis

The first analysis in structure, which included captive BBF wolves, resulted in  $K=4$  (Puechmaille 2016) and  $K=3$

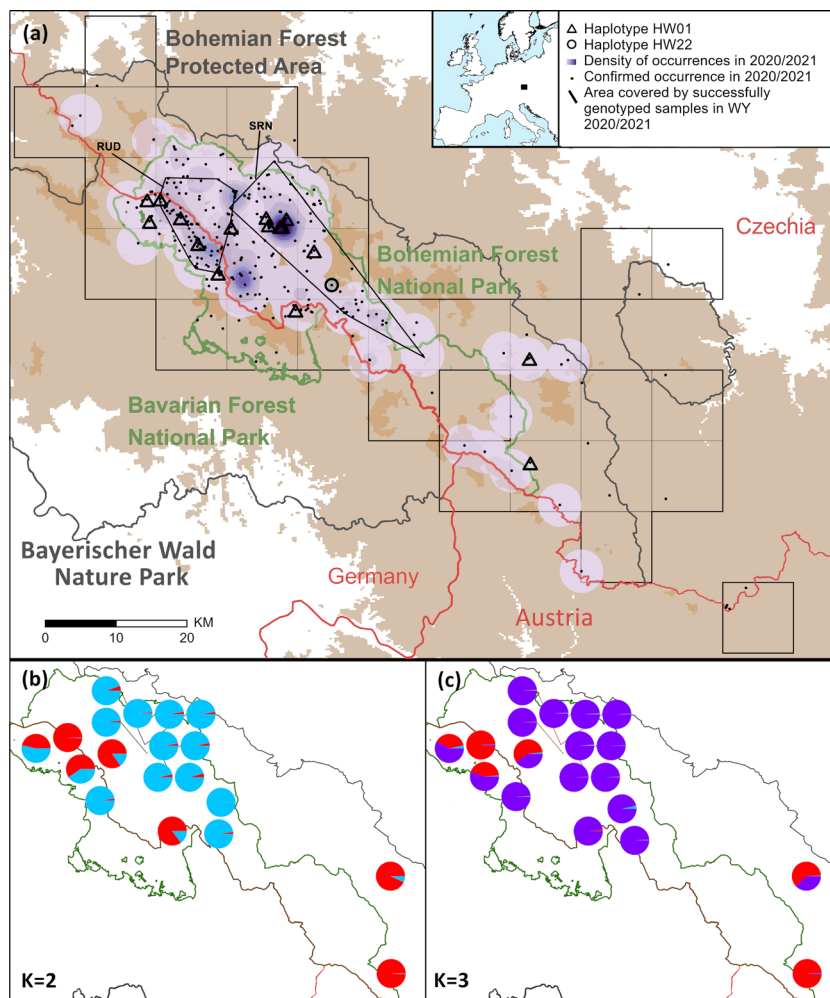


Figure 2. Sample localities and genetic affinities of admixed grey wolves in the Bohemian and Bavarian Forest in wolf years (WY) from WY 2013/2014 to WY 2020/2021. Genetic samples are displayed by the earliest detected occurrence of a particular individual. (a) Shows mitochondrial sequence haplotypes. Minimum convex polygons (black line) constructed from all genetic records of particular pack members in WY 2020/2021 visualise areas covered by successfully genotyped samples. The density map (purple colour scale) is constructed from all occurrence records (genetic and camera trap) in WY 2020/2021. Altitude is displayed in brown tones, scaled by 500 m with an altitude range of 0–1330 m. The EEA reference grid (10 × 10 km) is used. (b)–(c) Represent 13 nuclear microsatellite clustering, as detected by the Structure analyses when  $K=2$  (b) and  $K=3$  (c). The colour codes in (b) and (c) are as follows: Alpine population (blue), Central European pop. (red) and BBF deme (purple). Borders of protected areas are marked by green (national parks) and grey (other protected areas) lines. Borders of countries are marked by red lines.

(Evanno et al. 2005) being the best-supported number of clusters (Supporting information). Mixing between clusters of wild wolves and captive wolves from the enclosure was not observed. The second analysis, which included only wild wolves from BBF, suggested  $K=3$  (Puechmaile 2016) and  $K=2$  (Evanno et al. 2005) as the most likely number of clusters (Fig. 2b–c; Supporting information). In both wild BBF packs, an admixture of Alpine and Central European populations was detected. Results from the third analysis, which included only three individuals (offspring) of each pack in BBF, also suggested  $K=3$  (Puechmaile 2016) and  $K=2$  (Evanno et al. 2005) as the most likely number of clusters (Fig. 3a, Supporting information). In  $K=2$ , samples from the RUD pack showed higher admixture with the Central European population in comparison with samples from the

SRN pack. Results from FCA analysis show a clear division into three clusters (Fig. 3b–c). Admixture was confirmed also by analysis of mitochondrial DNA. Haplotype HW01 is predominantly found in the Central European population, while HW22 is characteristic of the Alpine population (Fig. 2a).

### Family relationships

Analysis of genealogies indicated the presence of two packs, Ruda (RUD) (in the west) and Srni (SRN) (in the east) (Fig. 2a, Supporting information). The density map in WY 2020/2021 is presented in Fig. 2a. Areas of polygons representing areas covered by successfully genotyped samples in WY 2020/2021 were 97 km<sup>2</sup> for the RUD pack and 185 km<sup>2</sup>

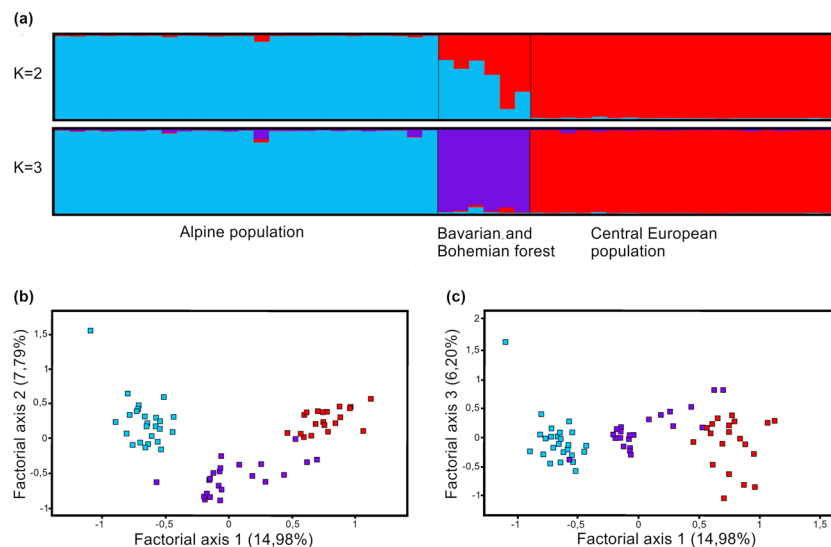


Figure 3. (a) Bayesian clustering analysis of the wild BBF wolf deme (three offspring per pack; six unique genotypes) and comparative Alpine ( $n=25$ ) and Central European ( $n=20$ ) populations using 13 microsatellite loci. The results are displayed for two ( $K=2$ ) and three ( $K=3$ ) clusters. Vertical lines represent individual genotypes, colour segments represent cluster membership coefficients ( $Q_i$ ), and colour codes correspond to Fig. 1. Full results of analysis (Delta  $K$  and Mean  $L(K) (\pm SD)$  according to Evanno et al. (2005), along with MedMedK, MedMeanK, MaxMedK and MaxMeanK statistics (Puechmaille 2016), are displayed in Supporting information. (b)–(c) Factorial correspondence analysis (FCA) of the wild BBF wolf deme ( $n=22$ ) and comparative Alpine ( $n=25$ ) and Central European ( $n=20$ ) populations using 13 microsatellite loci.

for SRN (Fig. 2a). The first established was the SRN pack. The breeding female (GW665f) bears haplotype HW01, and the breeding male (GW676m) bears HW22. The first capture of a genetic sample of the breeding male (GW676m) was ascertained in the Bavarian Alps, approximately 180 km away from the BBF region, measured as a straight-line distance (Supporting information). However, we were unable to detect the original pack of birth and there is a 3-year gap until genetic re-capture in BBF (Supporting information). The breeding female (GW665f) originated from the Central European population, with sparse occurrence in the Hercynian highlands (Szewczyk et al. 2021). Earlier occurrence of this individual was not detected, and thus the location of the birth pack of this female could not be determined, nor the distance she travelled.

So far, 14 offspring of the founder pack have been identified. A new pack close to Zelezná Ruda (RUD pack), with a male (GW1488m) and a female (GW1112f), was found. Based on parental analyses, the founding female of the RUD pack (GW1112f) was probably replaced by her sister (GW871f) during the sampling period. Both breeding females from the RUD pack were originally offsprings of the SRN pack (Supporting information). All three individuals were also bearing the HW01 haplotype. In both second-generation reproduction groups, two offspring were identified up until now. Long-distance dispersal of four male offspring from the SRN pack was detected: two individuals (GW942m and GW938m) dispersed to central and northern Germany (approximately 270 km and 520 km far, measured as straight-line distance), while one individual (CW20\_228m) was detected in Moravia (approximately 290 km far) and another

individual (GW1556m) dispersed into Austria (approximately 140 km far) (Supporting information).

## Discussion

### Genetically admixed deme

Due to the high mobility, elusiveness, and absence of distinctive coat patterns, genetic methods have proven to be the most efficient tool in assessing the population origins of wolf individuals (Hulva et al. 2018). The present study detected genetic admixture between the Alpine and Central European wolf populations (Fig. 2–3, Supporting information). BBF deme shows admixed genotypes at  $K=2$  and forms a third cluster in the case of  $K=3$ . We hypothesise that this pattern indicates the admixed nature of the deme in the BBF region. Bayesian clustering results and the presence of haplotypes that were not detected in the wild animals demonstrated that wolves held in captivity did not play a role in shaping the genetic composition of BBF deme. However, given the limited sample size, the results of Bayesian analyses should be interpreted with caution (Porrás-Hurtado et al. 2013). To validate and complement these conclusions, additional genomic data will be necessary to implement.

Wolves are characterised by the ability for both short-distance and long-distance dispersal, forming demes in more remote areas (Valière et al. 2003, Kojola et al. 2006, Jimenez et al. 2017, Jarausch et al. 2021). Regarding proximate mechanisms behind the BBF pack establishment, we hypothesise that admixture could occur in relation to the

Allee effect, i.e. that interbreeding between different forms could be facilitated by the low population density of at least one parental form in novel environments. This phenomenon frequently occurs in insular biogeography, including habitat islands and range offshoots (Courchamp et al. 2008).

While both BBF packs show clear evidence of admixture in the microsatellite analysis of nuclear DNA, the majority of the studied individuals bear the haplotype HW01, belonging to the Central European population. This can be explained by the maternal inheritance of mitochondrial DNA: only the first breeding male carried the haplotype HW22, unique to Alpine and Italian wolves. All progeny, including those of the second pack, inherited the HW01 haplotype from the female breeders, which originated from the Central European population.

Wolves of the Central European population are currently undergoing a rapid expansion within Central Europe (Hulva et al. 2018, Szewczyk et al. 2019, 2021, Jarausch et al. 2021). Alpine wolves are occasionally detected north of the Alps (Jarausch et al. 2021, own data). Therefore, crossbreeding between both populations is likely to continue. Although still speculative, we hypothesise that offspring from the admixed BBF packs could contribute to this process by forming a population deme in a geographically and ecologically intermediate region compared to parent populations (Hendricks et al. 2019). The detection of several wolves originating from these BBF packs, some even in quite distant regions, supports this hypothesis.

### Pack structure in Bohemian and Bavarian Forest

The first pack in the BBF region (SRN) was founded by long-distance dispersers, as the breeding male dispersed at least 180 km, measured as a straight-line distance. However, the unknown location of the original pack of birth, the 3-year gap in genetic re-capture in the BBF, and the landscape dispersal resistance (Huck et al. 2011, Ražen et al. 2016) suggest that this individual could have travelled a substantially longer distance before settlement. Previous studies have reported dispersal events of up to approximately 1200 km (Kojola et al. 2006, Andersen et al. 2015, Ražen et al. 2016, Byrne et al. 2018). Long-distance dispersal was detected in four offspring males from the SRN pack. These individuals travelled approximately 140–520 km from the original pack, measured as a straight-line distance. The BBF region may, therefore, represent an important source of expanding wolves in the future.

The sex ratio of wolves identified in the BBF region is strongly skewed towards males. Nevertheless, the sex ratio exhibits significant inconsistency across the studied packs in Europe (Kojola et al. 2006, Sidorovich et al. 2007, Stansbury et al. 2016, Jarausch et al. 2021).

In the RUD pack, the breeding female (GW1112f) was most likely replaced by a different individual. This female has been identified only twice in the past, and not again since the wolf year 2019/2020. She likely left or died; with a chance she remains in the area unidentified genetically.

In the scenario that the first female died, she was replaced by her sister (GW871f) the following year. This scenario is further supported by the results of parental analyses; in the year 2020/2021, two offspring of a replacement female and the founding male were genetically detected. This case is not unique, as similar cases have been reported. Brainerd et al. (2007) found that territorial wolves reproduced the following season in 47% of cases, with a greater proportion reproducing when one breeder had to be replaced (56%) compared to cases where both breeders had to be replaced (9%).

Although there is no supporting genetic evidence, the possibility of multiple reproduction and the potential of polygamy cannot be dismissed. Various studies provide evidence of multiple breeding females in larger packs (Mech and Boitani 2003, Ausband 2018, Sidorovich and Rotenko 2019, Ausband and Mitchell 2021), and several cases have been identified within the framework of legal genetic wolf monitoring in Germany (Carsten Nowak pers. comm.). The primary reason for multiple breeding was the mating of the dominant (founding) male not only with the founding female (mother) but also with daughters or other subdominant females. Moreover, empirical evidence indicates a polygamy threshold, with multiple breeding females observed mainly in groups with over eight individuals (Ausband 2018). However, the size of the RUD pack is estimated to be less than eight individuals. The potential process causing the aforementioned patterns involves polygamy in the second pack (SRN) with approximately 10 individuals estimated, but no multiple-breeding females have been observed in this pack so far. For this reason, and due to the lack of genetic evidence of a founding female from the RUD pack after the wolf year 2019/2020, we suppose that the possibility of death or the leaving of the first breeding female (GW1112f) is a more likely explanation.

The core areas of both BBF packs correspond to those of other studied packs from Europe, where the size of the territory varies between 100–400 km<sup>2</sup> (Fuller et al. 2003, Jędrzejewski et al. 2007, Duchamp et al. 2012). Overall, wolves exhibit high variability in their home range and territory sizes, strongly dependent on prey availability, latitude, and the stage of colonisation (Mattisson et al. 2013).

### Protected areas as stepping stone wilderness islands

Considering the national park status of the BBF, the study further illustrates the importance of protected areas as important tools in biodiversity conservation. They can preserve wildlife and provide habitats protected from disturbing human influence, especially poaching (Bassi et al. 2015, Rosenblatt et al. 2016, Henschel et al. 2020). In Germany, for instance, the initial wolf recolonisation has been facilitated by the settlement of packs in areas closed to the public, such as military training and mining areas (Reinhardt et al. 2019). While protected areas are often too small in Central Europe to serve as refugia for large carnivores, the Bavarian Forest and the Sumava National Parks span an area capable of hosting several wolf pack territories, and the number of packs



derived from the founding pair described above is increasing in recent wolf years. Therefore, they may display a region of particular importance for large carnivore conservation. This is further exemplified by the long-term preservation of a stable European lynx population in the BBF region (Müller et al. 2014) and points to the importance of large, protected nature regions as islands of relative wilderness within the densely populated anthropogenic landscapes of Central Europe. These regions can serve as stepping stones during the recolonisation process. The presence of apex predators, such as wolves, can, in turn, further increase the value of protected areas (Runte 2002) due to a keystone role and top-down effects on the ecosystem (Ripple and Beschta, 2012, Ripple et al. 2014).

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## Transparent peer review

The peer review history for this article is available at <https://publons.com/publon/10.1002/wlb3.01281>.

## Data availability statement

Information regarding mitochondrial haplotypes and sample coordinates are included in Table S1. Information regarding parental analysis is included in Table S2 and illustrated in Figure S6. Information about successful samples from the Bohemian and Bavarian Forest is available in Table S3. Information about microsatellite loci and their ranking in multiplexes are available in Table S4. Table S2–S5 and Figure S1–S6 are included in the Supporting information.

Primary data (microsatellite genotypes) together with Table S1 are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.zgmsbccdt> (Hulva et al. 2024).

## Supporting information

The Supporting information associated with this article is available with the online version.

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