

Recovery in the melting pot: complex origins and restored genetic diversity in newly established Eurasian beaver (*Rodentia: Castoridae*) populations

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Several larger vertebrate species have recovered from dramatic bottlenecks caused by overhunting and habitat destruction. One of the most notable comebacks concerns the Eurasian beaver (*Castor fiber* L.), which has increased its range by natural dispersal from both relict populations and populations established through translocations. Genetic methods have recently been used to study beavers at several locations. However, owing to a lack of reference samples from relict populations and alternative names of mitochondrial DNA haplotypes, the ancestry of re-established beaver populations remains obscure. Here, we focus on the genetic characterization of several newly established populations. Unlike previous studies, we also used microsatellite genotypes of reference samples from all relict populations. Our analysis was fully capable of tracing the origin of the nuclear and mitochondrial genome to relict populations. Although we confirmed an extraordinarily low genetic diversity in relict populations, our analysis showed restored diversity in newly established populations resulting from translocations. Recent expansions and stochastic effects have created a strong but complicated population structure, with neighbouring populations differing significantly in genetic composition. We conclude that this restored genetic diversity very likely contributes to the viability and ongoing expansion of the newly established populations.

ADDITIONAL KEYWORDS: conservation genetics – genetic ancestry – population recovery – spatial distribution.

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INTRODUCTION

Accelerating biodiversity loss, attributable to species extinction and ecosystem degradation, poses a significant threat at a global scale (Butchart *et al.*, 2010; Johnson *et al.*, 2017). Given that this loss of biodiversity can be ascribed largely to human activities, future crises can be expected in the human-dominated ecosystems of Europe. Surprisingly, however, many of the larger European mammal and bird species that had suffered dramatic bottlenecks owing to overhunting and habitat destruction have shown a return to viable population sizes in recent decades (Mucci *et al.*, 2010; Deinet *et al.*, 2013; Chapron *et al.*, 2014; Cortés-Avizanda *et al.*, 2015), with some (e.g. the Eurasian lynx, grey wolf, Eurasian otter, griffon vulture, white-tailed eagle and common crane) now even seen as flagship species of returning wilderness (Deinet *et al.*, 2013; Boitani & Linnell, 2015). The comeback of such species has been caused by several interacting factors, including species and habitat protection, farmland abandonment, reduced hunting pressure and, in some cases, translocations (Deinet *et al.*, 2013; Boitani & Linnell, 2015). Although translocations (movement and release into the wild) are an important tool in the conservation of endangered species (Allendorf & Luikart, 2007; O'Reilly *et al.*, 2021), they can also result in unintended negative outcomes for both the ecosystem and the target species (Novak *et al.*, 2021; Prendergast *et al.*, 2021). For example, negative outcomes for the translocated species include the risk of outbreeding depression, genetic swamping, disease transmission or loss of local adaptations (Allendorf *et al.*, 2010; Novak *et al.*, 2021). Moreover, many translocations pre-date and/or fail to follow genetically informed conservation measures with follow-on monitoring, resulting in populations of unknown origin and levels of mixture. Unravelling the mechanisms behind the comeback of species that previously faced extinction is crucial for the application of successful protection measures for other endangered species. In particular, a knowledge of the genetic structure of successfully recovering populations provides an important clue to an understanding of the processes governing population viability and subsequent changes in species abundance and range.

Perhaps the most astonishing comeback among the larger mammals and birds has been that of the largest Old World rodent, the Eurasian beaver (*Castor fiber* L.). Since 1955, the Eurasian beaver population has increased in abundance by > 14 000% and now almost completely covers its former continuous range, from the British Isles to eastern Siberia (Deinet *et al.*, 2013; Halley *et al.*, 2021). The return of the Eurasian beaver is unique among European mammals because it is characterized by a complex intermingled pattern

of both natural spread and human translocations, which has resulted in populations of mixed origin (Nolet & Rosell, 1998; Frosch *et al.*, 2014). The newly established populations exhibit high viability and tend to expand rapidly (Halley & Rosell, 2002; Halley *et al.*, 2012). As a result, this formerly endangered species can even be seen as a pest at newly colonized sites in human-dominated landscapes (Nolet & Rosell, 1998; Deinet *et al.*, 2013; Swinnen *et al.*, 2017).

Analysis of mitochondrial DNA (mtDNA) has shown that the two surviving beaver species, the North American beaver (*Castor canadensis* Kuhl, 1820) and the Eurasian beaver, diverged ~8 Mya (Horn *et al.*, 2011). Subsequently, the Eurasian beaver has spread across Eurasia, with the common ancestor of extant beavers dating back ~210 000 years (Horn *et al.*, 2011). The Eurasian beaver, a rare example of a genetically monogamous mammal, forms small family units comprising an adult breeding pair and offspring from the current and previous years (Willson, 1971; Campbell *et al.*, 2005; Müller-Schwarze, 2011; Syručkova *et al.*, 2015). All family members engage in delimitation of territory using scent marks (Wilsson, 1971; Rosell *et al.*, 1998). Within their territory, beavers typically use trees in riparian zones as building material for dams and lodges, and various parts of deciduous trees (e.g. the inner bark, leaves and twigs) make up a substantial part of their diet (Krojerová-Prokešová *et al.*, 2010; Vorel *et al.*, 2015; Janiszewski *et al.*, 2017; Lodberg-Holm *et al.*, 2021). As a result of this dietary specialization, it is likely that the once vast region that the beavers occupied during the Pleistocene shrank into refugia during glacial periods, when the opportunity to use deciduous trees as a dietary source was limited over large parts of Eurasia. The deep branching of extant mtDNA lineages suggests the existence of several refugia, one of which was probably situated on the Iberian Peninsula and others located more to the east (Ducroz *et al.*, 2005; Durka *et al.*, 2005). Moreover, analysis of ancient DNA has uncovered an additional mtDNA clade in the Danube Basin that went extinct and therefore did not contribute to the present-day genetic pool of Eurasian beavers (Horn *et al.*, 2014). The existence of separated western and eastern contemporary mtDNA lineages has led to the conclusion that the Eurasian beaver comprises at least two evolutionarily significant units (Durka *et al.*, 2005) and that this needs to be considered in management of beaver populations (Halley, 2011; but see Rosell *et al.*, 2012). However, it should also be noted that ancient DNA analysis suggests a potential overlap of the eastern and western groups in the past (Horn *et al.*, 2014), which is corroborated by the discovery of western group mtDNA haplotypes in eastern relict populations in Belarus and Voronezh in Russia (Senn *et al.*, 2014).

Not only have beavers traditionally been hunted for their castoreum (substances from the castor sacs), fur, meat and, occasionally, other parts of their body (Müller-Schwarze, 2011; Campbell-Palmer *et al.*, 2015), but also transformation of riparian zones into urban and agricultural landscapes (Petts, 1989) has led to substantial habitat loss, which has contributed greatly to a massive decline in beaver numbers (Veron, 1992). As a result, it is estimated that only 1200 individuals were surviving by the end of 19th century, with these being scattered in several different locations (Halley *et al.*, 2021). These ‘relict populations’ were located in the Rhone Delta (France), the middle Elbe (Germany), the Telemark region (South Norway), tributaries of the Dnieper and, perhaps, Neman rivers (Belarus, northern Ukraine and western Russia; hereafter referred to as the Belarus refuge), tributaries of the Voronezh and, probably, Don rivers (Russia), tributaries of the Irtysh and Ob rivers in Western Siberia (Russia), the Bulgan-Gol river (Ob river basin, Western Mongolia and Northwest China) and the Azas river in the Republic of Tuva (a tributary of the upper Yenissei, Russia) (Lavrov, 1981; Nolet & Rosell, 1998; Ducroz *et al.*, 2005; Durka *et al.*, 2005; Halley *et al.*, 2021). It is these relict populations that have acted as source populations for the current re-established Eurasian population (Nolet & Rosell, 1998). Beavers also survived at a few other sites, but these isolated populations probably went extinct (Lavrov, 1981; Veron, 1992) and therefore did not contribute to the present-day beaver gene pool. The relict populations differed considerably in structure and the number of surviving beavers, with small relict populations on the Azas river in Tuva or in the Rhone Delta in France probably harbouring only ~30 individuals, whereas the Belarus refuge, comprising several distant and, perhaps, isolated sites, probably harboured around ten times that number (Heidecke, 1986; Halley, 2011).

Beaver relict populations are characterized by a substantial reduction in genetic variability and alternatively fixed mtDNA haplotypes (Ellegren *et al.*, 1993; Millishnikov *et al.*, 1997; Babik *et al.*, 2005; Ducroz *et al.*, 2005; Durka *et al.*, 2005) and have traditionally been treated as separate subspecies owing to slight differences in morphology and disjunct distribution (Lavrov, 1981; Heidecke, 1986). However, the assignment into subspecies and usage of subspecies names has been rather inconsistent in the literature (reviewed by Gabrys & Wazna, 2003). Moreover, unique features in beavers from separate relict populations almost certainly originated only recently, owing to the strong drift that followed the severe bottleneck caused by human activities. Ancient beaver DNA does not provide any evidence of long-term isolation in relict populations that would support application of biologically meaningful

subspecies concepts (Reydon & Kunz, 2021), aside from the separation of the western and eastern clades (Horn *et al.*, 2014; Marr *et al.*, 2018). Additionally, during the recent recovery of the species, initiated by a combination of natural spread from relict populations and human translocations, beavers from the different relict populations have regularly come into contact (Dewas *et al.*, 2012; Frosch *et al.*, 2014).

Lavrov (1981) provided anecdotal evidence that beaver translocation attempts are likely to have started almost immediately after the severe decline; however, the first successful large-scale translocation took place in Sweden between 1922 and 1939, when ~80 individuals originating from southern Norway were released at 19 different sites (Curry-Lindahl, 1967; Hartman, 1995). Hundreds of translocations have followed throughout Eurasia, using various relict or re-established populations as a source. The return of beavers has been reinforced by natural expansion from both relict and re-established populations (Zahner, 1997; Schwab & Lutschinger, 2001; Halley, 2011; Dewas *et al.*, 2012; Halley *et al.*, 2021). As a result, the current estimated census size of the beaver population in Eurasia exceeds 1.5 million individuals (Halley *et al.*, 2021).

Translocations have often occurred repeatedly at the same sites, using individuals from different sources. For example, beavers from Norway, France, the former Soviet Union and Poland have all been reintroduced to into the Danube basin (Bavaria, Germany) since 1966 (Zahner, 1997; Schwab & Lutschinger, 2001; Frosch *et al.*, 2014), and the resulting admixed stock has since been used as a source for translocation programmes in Croatia, Hungary, Romania and Belgium (Schwab & Schmidbauer, 2001). Moreover, the North American beaver, which is difficult to differentiate from the Eurasian beaver in the field using morphological criteria (Danilov *et al.*, 2011; McEwing *et al.*, 2014), has been introduced or has escaped from captivity at several sites in Eurasia (Lahti & Helminen, 1974; Rossel *et al.*, 2005; Parker *et al.*, 2012). Although North American beavers have disappeared naturally at some sites, several translocations have resulted in viable populations, including large populations in Finland and north-western Russia (Parker *et al.*, 2012; Alakoski, *et al.*, 2019; Halley *et al.*, 2021). Owing to such repeated translocations and the secondary contact of expanding populations, the genetic composition of newly established populations remains unknown, even in situations where the origin of the reintroduced beavers was reported.

A number of recent studies have applied molecular tools to investigate beaver population ancestry and the level of admixture at sites in western and central Europe (Horn *et al.*, 2010; Kropf *et al.*, 2013; Biedrzycka *et al.*, 2014; Frosch *et al.*, 2014; Senn *et al.*, 2014; Minning *et al.*, 2016; Ernst *et al.*, 2017;

Mai *et al.*, 2018; Campbell-Palmer *et al.*, 2020; Iso-Touru *et al.*, 2020; Fedorca *et al.*, 2021), and these have consistently shown multiple source origins and a high level of admixture in most populations. Surprisingly, several new mitochondrial control region haplotypes, not known from relict populations, have been revealed in these newly established populations. However, it should be noted that some relict populations were not sampled properly in older studies (until the study by Senn *et al.*, 2014), and were therefore replaced by samples from neighbouring newly established populations. This concerns both the Voronezh relict population and the complex Belarus refuge. Given the almost simultaneous appearance of the latest studies, it is also likely that identical mtDNA haplotypes were reported under different names, which complicates further our understanding of population origin and genetic structure at a larger scale. Concerning nuclear loci, most studies lack reference samples from the relict populations; hence, the inference of the source origin based on nuclear loci remains rather speculative. Senn *et al.* (2014) used a panel of 306 single nucleotide polymorphism (SNP) markers and samples from relict populations; however, differentiation in the eastern relict population was rather poor, perhaps owing to ascertainment bias stemming from marker selection or relatively recent isolation of the relict populations.

Here, we use microsatellite loci and mtDNA markers that are thought to experience a more rapid mutation rate, thereby providing better discrimination of beaver relict populations. We compare the genetic diversity of the relict and newly established beaver populations and attempt to disentangle the origin and current genetic set-up of the newly established populations. In particular, we hypothesized that (1) the nuclear microsatellites would show greatly reduced genetic diversity in relict populations, in agreement with previously used markers; (2) the low genetic diversity of beavers in relict populations would have been restored in the newly established populations; (3) microsatellite loci would be differentiated clearly in relict populations; (4) microsatellite and mtDNA genetic markers would be capable of tracing the origin of nuclear and mitochondrial genomes in newly established populations back to relict populations; (5) the ascertained origin would correspond to reported translocations and natural dispersal; and (6) beavers of diverse origin would be shown to have admixed in the newly established populations.

MATERIAL AND METHODS

BEAVER POPULATIONS IN FOCUS

Samples from relict populations were described previously by Babik *et al.* (2005), Ducroz *et al.* (2005), Durka *et al.* (2005) and Senn *et al.* (2014), although

it should be noted that samples from some of the relict populations were not available until work of Senn *et al.* (2014). In earlier studies, beavers from the Belarus refuge were substituted with samples from neighbouring recently formed populations in Poland and Lithuania, whereas those from Voronezh (Russia) were substituted with samples from the relatively distant locality of Orël (representing the Oryol region; see Durka *et al.*, 2005: fig. 1). In contrast to these previous studies, we treat the localities in Poland and Lithuania as newly established populations (Table 1). Unfortunately, samples from Orël are no longer available; however, samples from the above-mentioned relict populations in Voronezh and Belarus later became available and appeared in study by Senn *et al.* (2014), and these are analysed here.

In addition to the above-mentioned newly established populations in Poland and Lithuania, we also examined newly established populations from the Kirov region of Russia and the Czech Republic in Central Europe (hereafter referred to as Czechia; Table 1). The beaver population in the Kirov region was formed by translocations from the Voronezh (24 individuals) and Belarus (51 individuals) relict populations in 1940 and 1954, respectively (Milishnikov & Saveljev, 2001). The samples from the Kirov region have previously been described and analysed by Senn *et al.* (2014). Beavers began naturally recolonizing Czechia from neighbouring countries around the end of the 1970s (Vorel *et al.*, 2012; Bartak *et al.*, 2013). The first newcomers originated from presumably admixed populations in Austria and Bavaria (Germany), and a second wave in the 1990s was the result of the Elbe River relict population expanding along the Elbe River (Vorel *et al.*, 2012). Beaver numbers were strengthened further by translocations between 1991 and 1997, when 27 individuals from Lithuania and north-east Poland were released in the eastern part of Czechia (Vorel *et al.*, 2012). The beavers originating from Bavaria and the Elbe River initially formed isolated populations in western and northern Czechia, respectively, whereas those originating from other sources (i.e. Austria and translocations) have formed a continuous large population in the eastern part of the country (Vorel *et al.*, 2012, 2016). Owing to the different origins of the populations and their present separation, we treat beavers from Czechia as belonging to three different populations (Table 1).

FIELD METHODS

Although much of the present study is based on the reanalysis of samples from previous studies, new samples were obtained from Czechia between 2004 and 2007 as part of a field monitoring and research project of the Czech University of Life Sciences in

Table 1. Diversity estimates of relict and newly formed Eurasian beaver (*Castor fiber* L.) populations based on 11 microsatellite loci

Population	Subspecies*	N	k	%PL	AR	GD	PA
Relict							
1. France	<i>C. f. galliae</i>	8	1.36	0.18	1.30	0.15	–
2. Germany	<i>C. f. albicus</i>	20	1.36	0.27	1.28	0.14	–
3. Norway	<i>C. f. fiber</i>	17	1.73	0.55	1.67	0.32	–
Belarus							
4. Grodno and Minsk region (Neman basin)	<i>C. f. belorussicus</i>	26	2.55	0.82	1.92	0.38	–
5. Gomel region (Dnieper basin)		1	–	–	–	–	–
6. Vitebsk region (Western Dvina basin)		7	1.82	0.64	1.76	0.33	–
7. Russia, Voronezh	<i>C. f. orientoeuropeus</i>	16	1.73	0.55	1.55	0.23	–
8. Russia, Western Siberia	<i>C. f. pohlei</i>	9	1.55	0.36	1.40	0.17	Cca92 (0.94, 0.06)
9. Mongolia	<i>C. f. birulai</i>	7	1.36	0.18	1.32	0.15	–
10. Russia, Tuva	<i>C. f. tuvinicus</i>	43	1.27	0.18	1.21	0.10	Cca62 (0.90), Cca56 (1.00)
Newly formed							
11. North Czechia (Elbe River)	–	15	1.55	0.45	1.36	0.17	–
12. Western Czechia	–	8	2.45	0.91	2.12	0.45	–
13. Eastern Czechia	–	40	2.82	0.91	2.17	0.45	–
14. Poland	–	7	2.45	0.73	2.10	0.44	–
15. Lithuania	–	34	2.91	0.91	2.14	0.45	Cca19 (0.02)
16. Russia, Kirov region	–	17	2.45	0.91	2.04	0.43	–

Estimates are not given for the Gomel region in Belarus owing to small sample size.

*Usage of subspecies names for beavers in Eastern Europe is inconsistent in the literature and would require deeper taxonomic revision.

Abbreviations: AR, allelic richness; GD, gene diversity; k, number of alleles per locus; N, number of individuals; PA, loci harbouring private alleles (with the allele frequency in parenthesis); %PL, proportion of polymorphic loci.

Prague (Table 1). In this case, beavers were trapped at night using Hancock live traps, and 1.5 mL of blood was collected from the medial saphenous vein of the hindlimb using 0.8 mm × 25 mm needles and EDTA vacuum tubes. The blood samples were then kept frozen at –20 °C until analysis. After sampling, the beavers were released at the site of capture. DNA was extracted using the DNeasy blood and tissue kit (QIAGEN), according to the manufacturer's protocol.

MITOCHONDRIAL DNA

Samples from Czechia were sequenced for the mtDNA control region using the primers Thr-L15926 and DL-H16340, in conditions described by Durka *et al.* (2005). Sequences of extant beavers from previous studies were downloaded from GenBank (see Supporting Information, Table S1) and aligned in BIOEDIT v.7.2.5 (Hall, 1999) using implemented CLUSTAL W (Thompson *et al.*, 1994). The alignment was trimmed to 489 bp and collapsed to haplotypes in FABOX v.1.5 (Villesen, 2007). Relationships between haplotypes of relict populations were visualized using the median-joining method (Bandelt *et al.*, 1999) in POPART (Leigh & Bryant, 2015).

MICROSATELLITE LOCI

We genotyped 275 individuals at 11 microsatellite loci (Supporting Information, Table S2) originally designed for the North American beaver, i.e. Cca4, Cca5, Cca8, Cca13, Cca18 and Cca19 from Crawford *et al.* (2008), and Cca20, Cca56, Cca62, Cca76 and Cca92 from Pelz-Serrano *et al.* (2009). Redesigned primers were used for amplification of Cca56, Cca62, Cca76 and Cca92, as described by Syručkova *et al.* (2015). Basic characteristics of loci are also given by Syručkova *et al.* (2015). We experienced serious problems with amplification of the Eurasian beaver microsatellite loci described by Frosch *et al.* (2011), especially when working with older DNA samples. It should also be noted that the selection of loci described by Frosch *et al.* (2011) was based on polymorphism in only a few Eurasian beaver populations, which might have introduced ascertainment bias, as shown by Senn *et al.* (2014) (see also Discussion). For this reason, we limited our analysis to the set of 11 loci mentioned above.

The primers were fluorescently labelled, and the loci were amplified in a single multiplex polymerase chain reaction (PCR). The PCR thermal profile comprised an initial 5 min run at 95 °C, followed by 30 cycles of 30 s at 95 °C, 90 s at 56 °C and 30 s at 72 °C, with the run being terminated by a final extension of 30 min at 60 °C. The

PCR products were mixed with Gene Scan 500LIZ size standard (Applied Biosystems) and subsequently run on an ABI PRISM 3130 Genetic Analyser. Genotypes were scored using GENEMARKER v.1.9 software (Softgenetics).

POPULATION GENETIC ANALYSIS

The number of alleles per locus, gene diversity and allelic richness for each population were calculated using FSTAT v.2.9.3.2 (Goudet, 2001). Allelic richness was standardized using the rarefaction method, with a minimum sample size of three individuals. One-sided permutation tests in FSTAT were used to compare allelic richness, gene diversity and F_{ST} (the fixation index, a measure of population differentiation) between relict and newly established populations, with P -values obtained after 10 000 permutations. Tests of linkage disequilibrium (LD) and deviation from Hardy–Weinberg equilibrium were carried out in the online version of GENEPOP (Raymond & Rousset, 1995; <http://genepop.curtin.edu.au/index.html>), using default values for Markov chain parameters. GENALEX v.6.51 (Peakall & Smouse, 2006, 2012) was used for inferring the number of private alleles in each population, estimating molecular variance within and between populations (analysis of molecular variance) and calculating the pairwise F_{ST} for each pair of populations. The genetic relationship between populations was visualized using a NEIGHBOURNET split graph constructed in SPLITSTREE4 (Huson & Bryant, 2006). Genetic distances used in construction of the graph were calculated from microsatellite data using the method of Smouse & Peakall (1999), as implemented in GENALEX v.6.51 (codom-genotypic option).

The Bayesian clustering method implemented in STRUCTURE v.2.3.3 (Pritchard *et al.*, 2000) was used to assign individuals into groups, using the following settings: K (the assumed number of groups) ranging from one to 15, with 800 000 iterations, 200 000 steps of burn-in and 30 independent runs for each K . Default values were used for all other parameters. The results of each run were processed using CLUMPAK (Kopelman *et al.*, 2015), utilising the CLUMPP (Jakobsson & Rosenberg, 2007) and DISTRUCT (Rosenberg, 2004) software packages. Optimal K was determined in KFNINDER v.1.0 (Wang, 2019) using mean $\Pr[X|K]$ (the

probability of genotype data X given K) (Pritchard *et al.*, 2000), DeltaK (Evanno *et al.*, 2005) and the parsimony index method (Wang, 2019).

RESULTS

GENETIC DIVERSITY AND DIFFERENTIATION OF POPULATIONS

For the 11 microsatellite loci examined, we did not find any consistent significant LD between pairs of loci across populations, and there was no consistent deviation from Hardy–Weinberg equilibrium across populations at any locus. Analysis of molecular variance revealed that population structure (i.e. assignment of individuals into populations) explained > 50% of variation in the microsatellite data (Table 2). Overall differentiation ($F_{ST} = 0.516$) and pairwise F_{ST} values (mean = 0.51, SD = 0.22 and range = 0.02–0.86; Supporting Information, Table S3) were high and, in all but one case (between the two Elbe River populations), significantly different from zero. Although allelic richness ($P = 0.007$) and gene diversity ($P = 0.039$) were significantly lower in relict populations than in newly established populations (Table 1), F_{ST} was higher ($P = 0.047$) in relict populations ($F_{ST} = 0.654$) than in newly established populations ($F_{ST} = 0.298$).

Most relict populations were clearly differentiated over a large range of K in the STRUCTURE analysis (Figs 1, 2), with two exceptions being the relict populations from western Siberia (Russia; *Castor fiber pohlei*) and Mongolia (*Castor fiber birulai*), which were clearly separated at $K = 2$ and 3 but appeared in a single cluster at $K > 6$. All three methods for optimal K estimation gave relatively high values (DeltaK = 9, parsimony index method = 10, $\Pr[X|K] = 12$); however, the results showed clear hierarchically nested structuring at $K > 7$, in which the structure of the relict populations remained constant. The main difference in assigning individuals at $K > 7$ concerned beavers from the Belarus refuge and the newly established populations to which this refuge might have contributed. For this reason, we used $K = 8$ in figures showing the genetic composition of beaver populations (Figs 2, 3), this being the lowest K at which relict populations were clearly differentiated and which provided clear biological interpretation.

Table 2. Analysis of molecular variance summary for microsatellite loci

	d.f.	SS	Total variance (%)	F_{ST}	P -value
Among populations	13	869.769	52	0.516	0.001
Within populations	536	862.020	48	–	–

Abbreviations: F_{ST} , the fixation index, a measure of population differentiation; SS, sum of squares.

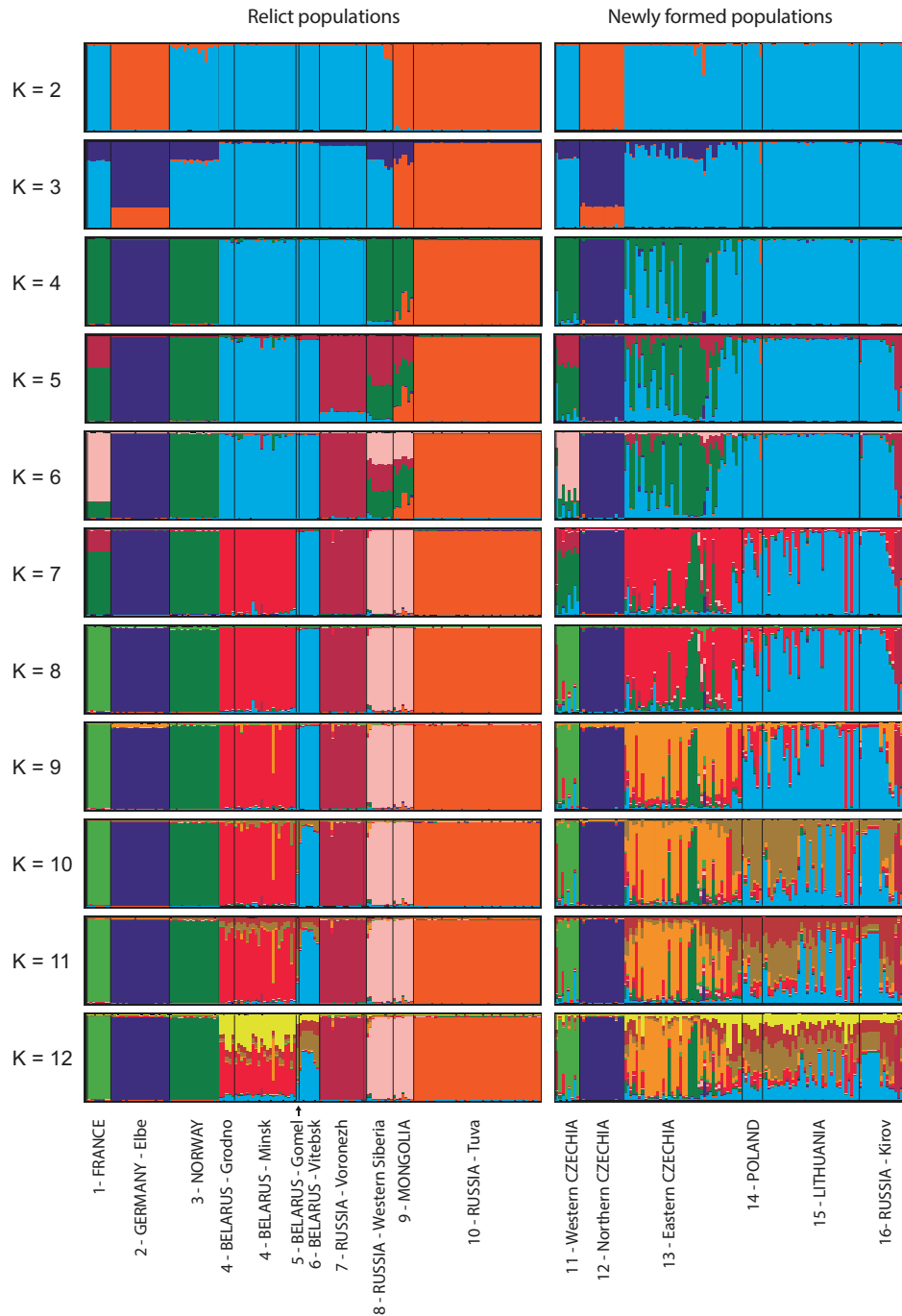


Figure 1. Results of STRUCTURE analysis based on microsatellite data for values of K (the number of assumed groups) ranging from two to 12.

Beavers from the Belarus refuge were assigned into two clusters at $K > 6$, with the main division being western localities in the Grodno and Minsk regions (Neman water basin) vs. north-eastern localities in the Vitebsk region (western Dvina water basin) and the south-eastern Gomel region (Dnieper water basin), the latter two being separated at $K = 10$.

The results of STRUCTURE analysis were corroborated by the NEIGHBOURNET network based on nuclear loci (Fig. 4), which clearly separated relict populations. The network showed a close relationship between most eastern relict populations from Mongolia and Tuva (Russia); however, it also supported a close relationship between populations from Mongolia and

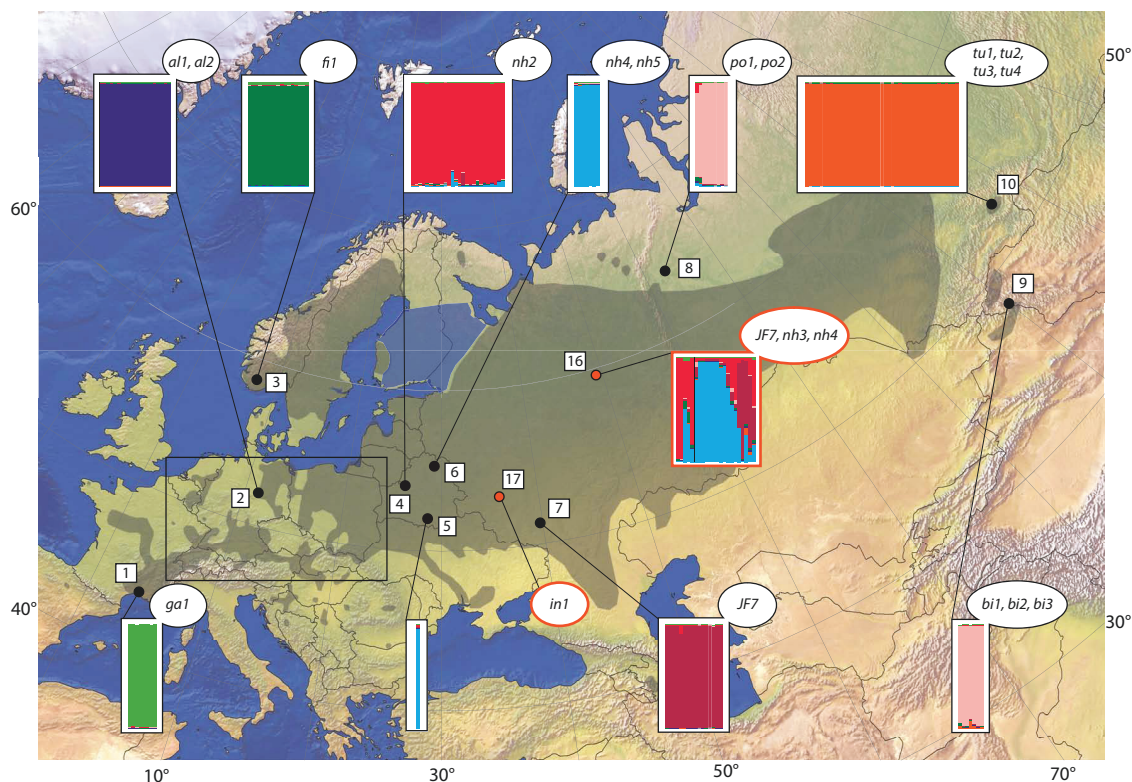


Figure 2. Genetic structure of Eurasian beaver populations in Eurasia. Eurasian beaver distribution (indicated by grey shading) is given as in the paper by Halley *et al.* (2012), which corresponds roughly to the time of sampling. The present-day distribution differs slightly in terms of possible contact between relict and introduced populations (see main text for details). The distribution of introduced North American beavers in Finland and Russia is shown by the blue shading. Both control region mitochondrial DNA haplotypes from the literature and STRUCTURE results ($K = 8$) based on microsatellite data are shown. Samples from relict populations are indicated by black dots and frames, and newly established populations are indicated by the red shading. DNA samples from the locality in the Oryol region of Russia (harbouring the *in1* mitochondrial DNA haplotype) were not available for microsatellite analysis.

western Siberia (Russia), which was also suggested by STRUCTURE analysis. In contrast to the STRUCTURE analysis, the single beaver sample from the Gomel Region in Belarus appeared to differ genetically from beavers at other Belarus localities. Overall, the clear separation into western and eastern clades found in previous studies based on mtDNA (see also our mtDNA results below) was not so clear in the microsatellite NEIGHBOURNET network.

The mtDNA haplotype network (Fig. 5) supported the division of haplotypes into western and eastern clades suggested in previous studies. In agreement with Senn *et al.* (2014), haplotypes from the Belarus refuge occurred in both clades, with haplotype *nh5* from Vitebsk and *nh2* from Minsk and Grodno belonging to the eastern clade, and the *nh4* haplotype, which was also found in Vitebsk, clustering with western haplotypes. Haplotype *JF7* from Voronezh, which was widely distributed across newly established populations, clearly fell into the western clade.

ORIGIN AND ADMIXTURE OF BEAVERS IN NEWLY FORMED POPULATIONS

In contrast to relict populations, the newly established populations showed high levels of mixture and admixture (Figs 1–3). Nevertheless, genetic origin (assignment to a combination of relict populations) could be ascertained unequivocally in the STRUCTURE analysis at $K > 6$ and was compatible with the origin of corresponding mtDNA haplotypes. The inferred origin and level of admixture differed greatly between newly established locations and formed population-specific genetic signatures, which was also reflected by a relatively high F_{ST} (0.298). In particular, geographically close localities in Central Europe showed enormous genetic dissimilarity.

The sample from the Elbe River in northern Czechia was assigned unequivocally to the Elbe River relict population in Germany, whereas the sample from western Czechia was affiliated mainly with a cluster comprising the French relict population, with minor

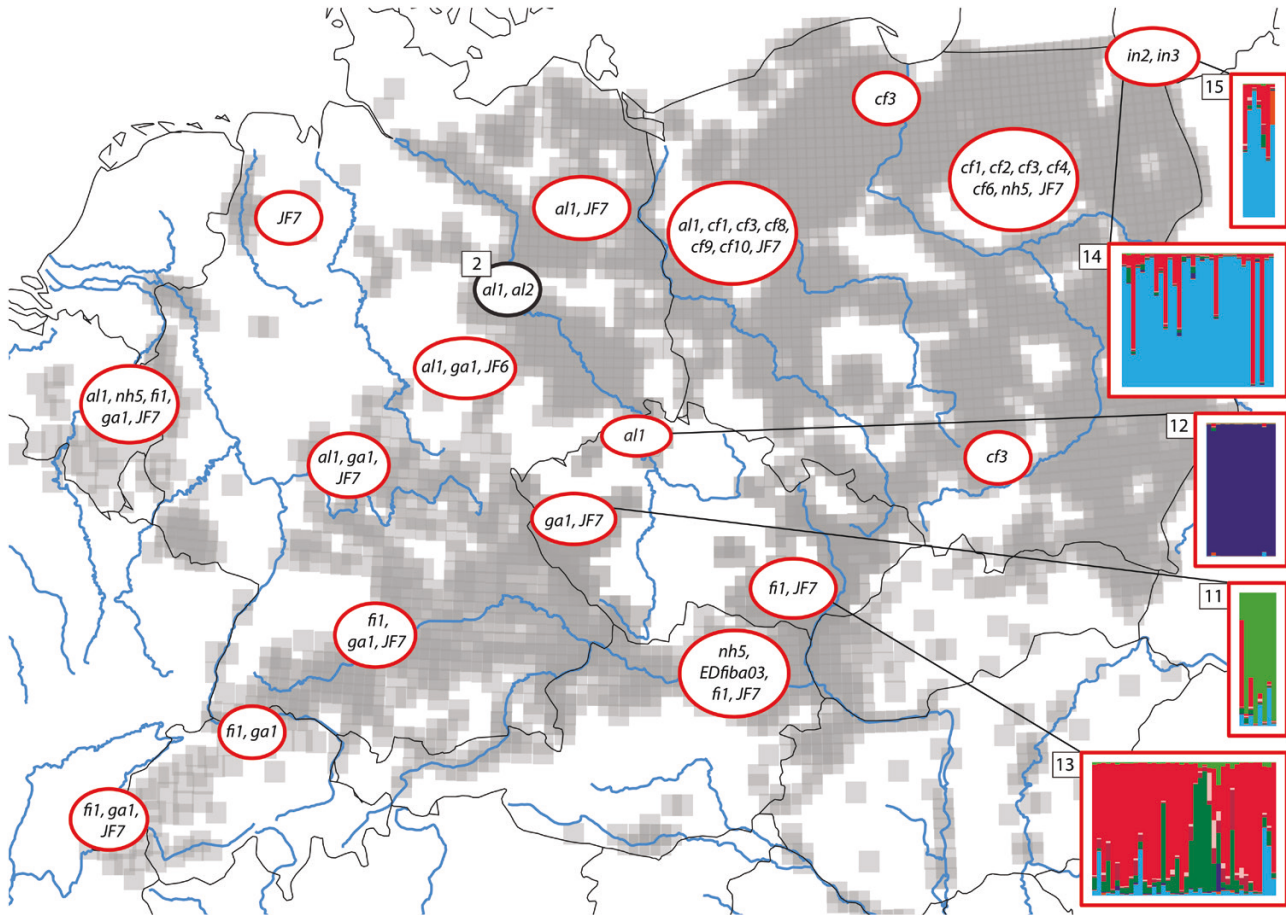


Figure 3. Detailed overview of the genetic structure of Central European beaver populations. Both control region mitochondrial DNA haplotypes and STRUCTURE results ($K = 8$) based on microsatellite data are shown.

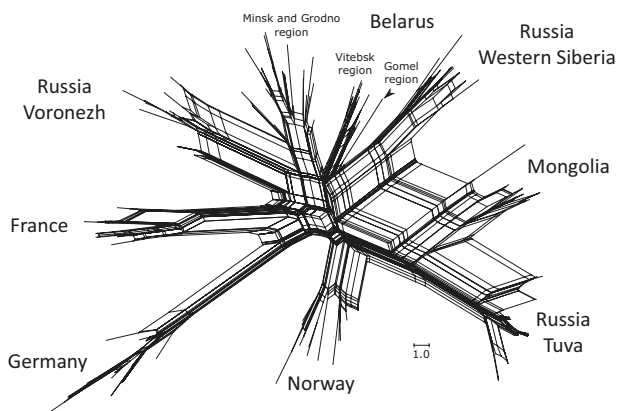


Figure 4. Relationships between beavers from relict populations, shown using a NEIGHBOURNET network based on microsatellite data.

admixture from both main Belarus groups. The sample from eastern Czechia (Moravia) was dominated by a signal from the Belarus relict populations, but also

indicated a contribution from Norwegian beavers. It should be noted, however, that at $K > 8$, the western Belarus relict population and most beavers from eastern Czechia (red cluster in Fig. 1) were separated into two separate clusters (red and orange in Fig. 1), with the orange cluster dominating in eastern Czechia but having only weak representation in western Belarus. This might suggest that the exact locality in the Belarus refuge to which the orange group corresponds was not included in our study, or it might be explained by shifts in allele frequency owing to a founder effect associated with range expansion. A similar effect appeared in the samples from Poland and Lithuania at $K > 9$; however, at lower values of K , the samples from Poland and Lithuania showed a dominant contribution from the eastern Belarus group and a minor contribution from the western Belarus groups. The sample from the Kirov region showed substantial substructuring, with beavers sampled west of Kirov largely corresponding to a western Belarus origin, whereas beavers trapped further east were characterized by a stronger signal indicating a Voronezh origin.

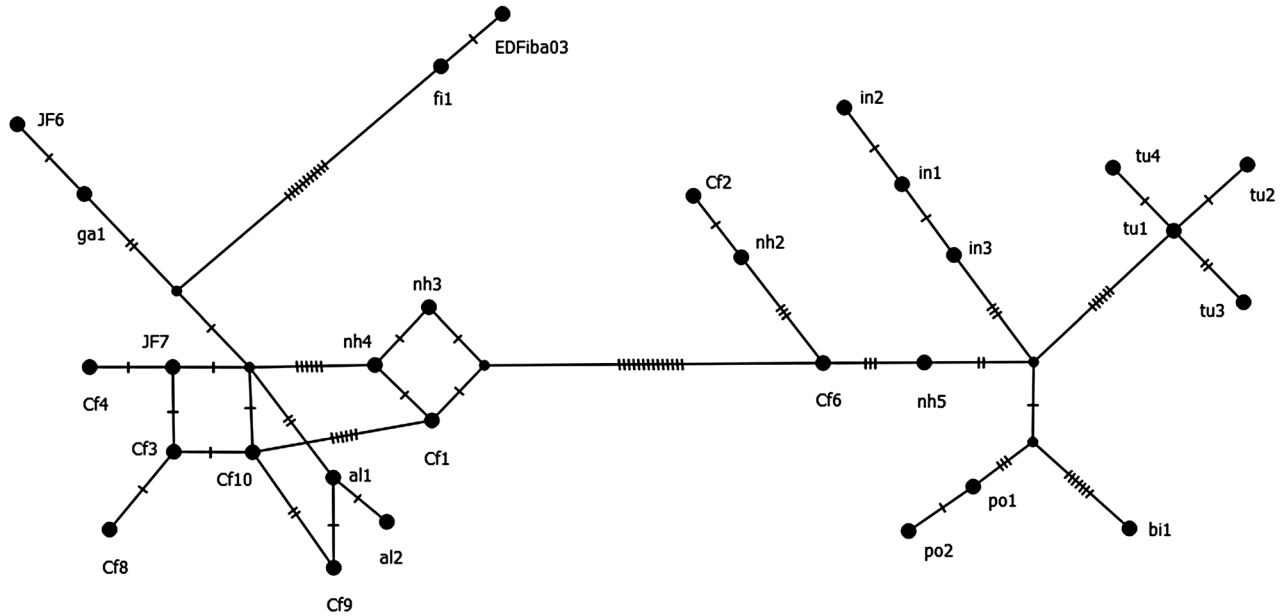


Figure 5. Median-joining network for mitochondrial DNA control region haplotypes of extant beavers. Note that haplotypes *bi2* and *bi3* differ by indels from *bi1* (Durka *et al.*, 2005) and are therefore not separated in the network.

Table 3. Alternative names of mitochondrial control region haplotypes

Haplotype	Alternative name(s)	References
<i>JF7</i>	<i>JF264887</i> , <i>COL00024</i> , <i>r1</i> , <i>Cf5</i>	Horn <i>et al.</i> (2010); Kropf <i>et al.</i> (2013); Biedrzycka <i>et al.</i> (2014); Frosch <i>et al.</i> (2014); Senn <i>et al.</i> (2014)
<i>nh5</i>	<i>COL00025</i> , <i>e</i> , <i>Cf7</i>	Kropf <i>et al.</i> (2013); Biedrzycka <i>et al.</i> (2014); Frosch <i>et al.</i> (2014); Senn <i>et al.</i> (2014)
<i>nh4</i>	<i>r2</i>	Frosch <i>et al.</i> (2014); Senn <i>et al.</i> (2014)
<i>nh3</i>	<i>r3</i>	Frosch <i>et al.</i> (2014); Senn <i>et al.</i> (2014)
<i>fi1</i>	<i>COL00027</i> , <i>f</i> , <i>fi</i>	Durka <i>et al.</i> (2005); Kropf <i>et al.</i> (2013); Frosch <i>et al.</i> (2014); Mai <i>et al.</i> (2018)
<i>JF6</i>	<i>JF264886</i> , <i>ger4</i>	Horn <i>et al.</i> (2010); Biedrzycka <i>et al.</i> (2014); Senn <i>et al.</i> (2014)
<i>al1</i>	<i>a1</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>al2</i>	<i>a2</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>bi1</i>	<i>b1</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>bi2</i>	<i>b2</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>bi3</i>	<i>b3</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>ga1</i>	<i>g</i> , <i>ga</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014); Mai <i>et al.</i> (2018)
<i>in1</i>	<i>i1</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>in2</i>	<i>i2</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>in3</i>	<i>i3</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>po1</i>	<i>p1</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>po2</i>	<i>p2</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>tu1</i>	<i>t1</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>tu2</i>	<i>t2</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>tu3</i>	<i>t3</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)
<i>tu4</i>	<i>t4</i>	Durka <i>et al.</i> (2005); Frosch <i>et al.</i> (2014)

Concerning the published mtDNA haplotypes retrieved from GenBank, our analysis revealed frequent usage of alternative names for identical haplotypes

(Table 3). Haplotypes in newly established populations were largely identical with those in relict populations or differed by a single substitution from a relict population

haplotype (Fig. 5). Two haplotypes from Poland (*Cf8* and *Cf10*) differed by four substitutions from relict haplotypes *JF7* and *al1* but were well integrated into the cluster of haplotypes from the western part of Eurasian beaver distribution. In contrast, there was a well-separated cluster of *in1*, *in2* and *in3* haplotypes from Poland, Lithuania and Orël in Russia that was not found in any of the relict populations. We also obtained new mitochondrial control region sequences for 64 individuals from newly established populations in Czechia (Fig. 4). All 17 sequenced individuals from the northern Elbe River population possessed the *al1* haplotype, which was also the most frequent haplotype in the Elbe relict population in Germany (Durka *et al.*, 2005). Haplotypes *ga1* (found in 12 individuals) and *JF7* (one individual), which correspond to relict populations in France and Voronezh, respectively, were also found in western Czechia, whereas individuals in eastern Czechia possessed haplotypes *fi1* (34 individuals) and *JF7* (ten individuals), suggesting a Norwegian and Voronezh origin.

DISCUSSION

RESTORED GENETIC DIVERSITY OF NEWLY ESTABLISHED POPULATIONS

We were able to demonstrate that translocation of beavers from different source populations has restored diversity in recently formed populations. This was especially clear in newly established Central European populations. These populations function as ‘melting pots’, wherein beavers from several distant lineages meet and begin to admix. Although some translocations in other species have certainly resulted in positive outcomes for the focus species and the surrounding habitat, others have had unintended negative consequences (Allendorf & Luikart, 2007; Novak *et al.*, 2021). Some translocated populations experience decreased genetic diversity (Furlan *et al.*, 2020), which can put their survival at risk (Schäfer *et al.*, 2021; DeWoody *et al.*, 2021). In the case of beavers, however, numerous studies have described positive effects from beaver reoccurrence on both habitat and species diversity (e.g. Ciechanowski *et al.*, 2011; Law *et al.*, 2017; Kivinen *et al.*, 2020; Nummi & Holopainen, 2020; Thompson *et al.*, 2021). Moreover, in the present study, in agreement with Frosch *et al.* (2014), we propose that the restored genetic diversity has very probably contributed to the viability and ongoing expansion of the newly established beaver populations.

DIFFERENTIATION AND DIVERSITY OF RELICT POPULATIONS

Despite being based on a limited number of microsatellite loci, our analysis clearly differentiated

most of the relict populations and allowed for unequivocal assignment of individuals to these relict populations. Surprisingly, we obtained a better resolution than a previous analysis based on 306 SNP markers (Senn *et al.*, 2014), which failed to separate several (mainly eastern) relict populations. It should be noted, however, that marker selection by Senn *et al.* (2014) was based on a comparison of only two beaver populations from Bavaria and Norway, which, despite careful marker selection, probably introduced an unknown amount of ascertainment bias into the analysis (Senn *et al.*, 2013, 2014).

Aside from the Belarus refuge, the relict populations were characterized by extraordinarily low diversity, high differentiation and a low proportion of polymorphic loci, which is in good agreement with previous genetic studies (Ellegren *et al.*, 1993; Babik *et al.*, 2005; Ducroz *et al.*, 2005; Durka *et al.*, 2005) and supports a very low estimated population size during the bottleneck (Halley, 2011). In contrast, our analysis suggests that the Belarus refuge population is relatively complex, perhaps unsurprisingly, given that it is one of the largest relict populations and spreads across several water basins. Both STRUCTURE analysis at $K > 6$ and the NEIGHBOURNET network based on microsatellite data indicated a clear division of individuals into at least two groups, suggesting that beavers survived the bottleneck in this area at several isolated localities. Although samples from the Neman Basin (Grodno and Minsk regions) conclusively form a coherent group, the relationship of beavers from the Vitebsk and Gomel regions (western Dvina and Dnieper basin, respectively) requires further examination.

Mongolian and western Siberian beavers (*C. f. pohlei* and *C. f. birulai* subspecies) were not clearly differentiated in some analyses. Although this suggests that STRUCTURE analysis based on a limited number of markers was inadequate in this case, we cannot fully exclude the possibility that the two distant relict populations were in contact in the past, because both are situated within the Ob basin. On the contrary, the close relationship between the Mongolian and Tuva relict populations observed in the NEIGHBOURNET network appears to fit their geographical positions better.

STRUCTURE AND ORIGIN OF NEWLY ESTABLISHED POPULATIONS

We found that most of Europe and Asia is now populated by beavers of very complex origin. The genetic structure of European mammal populations has been shaped by postglacial colonizations from multiple refugia, with some of those situated in the north being cryptic (Kotlik *et al.*, 2006; Lebarbenchon *et al.*, 2010; Wojcik *et al.*, 2010; Swenson *et al.*, 2011;

Schmitt & Varga, 2012; Ruiz-González *et al.*, 2013; Markova *et al.*, 2020). This expansion of different refugial lineages resulted in a series of complicated Central European contact zones (Randi *et al.*, 2003; Mucci *et al.*, 2010; Bolífková & Hulva, 2012; Bolífková *et al.*, 2017; Hulva *et al.*, 2018; Zolotareva *et al.*, 2021). Moreover, the genetic structure has also been modified by bottlenecks (Frantz *et al.*, 2014; Ratkiewicz *et al.*, 2014) and stochastic effects at the expansion front (Austerlitz *et al.*, 1997; Excoffier & Ray, 2008; Excoffier *et al.*, 2009; Hagen *et al.*, 2015). The situation becomes even more complex in populations that have been strengthened or were founded by translocations. In such populations, the original genetic structure can become blurred as new patterns originate owing to stochastic genetic capture (Wright *et al.*, 2014). The genetic structure of beaver populations appears to be the result of a mixture of all the above-mentioned processes, the effects of which are almost impossible to separate. Unlike other mammals, however, the role of translocations appears to have been essential in shaping the genetic composition of populations.

The situation in Central Europe merits special attention. Here, a complex translocation and expansion history has created a uniquely strong population structure, with nearby locations differing significantly in origin and genetic composition. Genetic theory and empirical studies (Austerlitz *et al.*, 1997; Excoffier *et al.*, 2009; Hagen *et al.*, 2015), however, suggest that this might be only a transient phase, with such differences eventually being erased as extensive contact between populations results in a general homogenization and overall loss of population structure.

The genetic origin of newly established populations can be deduced from microsatellite data, which was not possible in previous studies owing to a lack of reference samples from relict populations or lack of resolution in the genetic markers used. The Czech Elbe population showed a clear affiliation to the Elbe River relict population in Germany, with no admixture with beavers from other sources. However, it should be noted that both Elbe samples were obtained > 10 years ago and that the present-day situation might differ, as shown by Frosch *et al.* (2014). Recently, both Elbe populations (German relict and Czech newly established) have come into contact with other populations, and other newly established populations already show a substantial level of admixture, which is in agreement with previous microsatellite studies from Poland, Germany, Switzerland, Luxembourg and Belgium (Biedrzycka *et al.*, 2014; Frosch *et al.*, 2014; Mai *et al.*, 2018). In all newly established populations, however, there are some individuals that show very little (if any) admixture, and some newly established populations show clear signs of local substructuring (e.g. the Kirov site). This pattern probably stems

from the recent origin of populations, together with the long generation time of beavers (beavers do not attain sexual maturity until their second year) and the delayed dispersal of subadults (subadults remain with the family and help in rearing young) (Hinze, 1960; Wilsson, 1971; Mayer *et al.*, 2017). An alternative explanation might be that, in otherwise admixed populations, selection maintains large co-adapted genome blocks in some individuals (Franklin & Lewontin, 1970; Hedrick 2013; Sachdeva & Barton, 2018), although this seems less likely. We also cannot exclude the effect of behavioural subspecies discrimination, perhaps based on olfactory cues (using castoreum scent marks), which has been reported previously in Eurasian beavers (Rosell & Steifetten, 2004). However, we can only speculate on whether such discrimination would lead to assortative mating in newly established populations.

DISPUTABLE UNSAMPLED CRYPTIC RELICT POPULATIONS

At $K > 8$, STRUCTURE analysis indicated a considerable number of individuals in the newly established populations in Czechia, Poland, Lithuania and the Kirov region displaying affiliation to a group not covered by our relict population sample (orange cluster in Fig. 1). At lower values of K , however, these individuals were affiliated with the western Belarus relict population. Here, we provide two non-mutually exclusive explanations for these patterns. On the one hand, newly established populations might have experienced strong demographic founder effects associated with translocations, causing allele frequencies to shift and complicating correct assignment to a relict population. On the other hand, we were able to demonstrate that the large Belarus refuge was spatially complex, with genetically differentiated subpopulations. It is possible that the refuge population was inadequately sampled, meaning that the divergent clusters in the newly established populations (orange and brown clusters in Fig. 1) correspond to unsampled parts of the refuge. This latter hypothesis appears to be supported by the Polish and Lithuanian *in2* and *in3* mtDNA haplotypes (Durka *et al.*, 2005), which were not found in any relict population. Surprisingly, the *in2* and *in3* haplotypes were not found in a recent study by Biedrzycka *et al.* (2014), who sequenced 65 individuals from different regions of Poland. Hence, we cannot exclude the alternative possibility that the control region haplotype results were obscured by the presence of nuclear pseudogenes (mutated copies of mtDNA sequences incorporated into nuclear DNA that might be amplified and sequenced instead of mtDNA), as seen in other rodents (DeWoody *et al.*, 1999; Triant & DeWoody, 2008; Filipi *et al.*, 2015). It should be

noted, however, that nuclear pseudogenes were not found in the study by Horn *et al.* (2011), who mapped the complete beaver mitochondrial genome. Given that native beavers were reported from eastern Poland until the end of World War II (Dzieciolowski & Gozdziwski, 1999), we can speculate that the unsampled relict (sub)population was possibly situated in eastern Poland or adjacent parts of Lithuania. The situation might be even more complicated, however, because there were several poorly documented translocations in the Neman water basin before World War II (Lavrov 1981). The existence of unsampled relict populations was also suggested by Biedrzycka *et al.* (2014) based on the haplotype diversity found in Poland; however, it should be noted that this diversity could be explained, at least in part, by translocations from several relict populations. Some of the haplotypes in the study by Biedrzycka *et al.* (2014) are identical or very similar to haplotypes recently found in relict populations.

Recently published analyses of newly established populations have reported the existence of a number of new mtDNA haplotypes that appear to be absent in relict populations. However, several of these haplotypes were simultaneously being used under different synonyms, owing, in part, to the almost coincidental publication of three papers in the same year (i.e. Biedrzycka *et al.*, 2014; Frosch *et al.*, 2014; Senn *et al.*, 2014). Revision of the control region mtDNA haplotypes found counterparts in relict populations for most haplotypes found in the newly established populations. After synonymization, the distribution of haplotypes fitted well with known beaver translocation history. Perhaps the most important of these was the synonymization of the *JF7* haplotype, which had several alternative names and was present in most newly established populations. A Russian origin of *JF7* was correctly predicted by Frosch *et al.* (2014), where it was referred to as *r1*, and it was found simultaneously in the Voronezh relict population by Senn *et al.* (2014). The *nh5* haplotype, which originated from the Belarus (Vitebsk) relict population, also appears to be widespread. This haplotype is identical with COL00025 in the paper by Kropf *et al.* (2013), who erroneously assigned it to *C. f. pohlei* from western Siberia, causing further confusion in the literature (Saveljev & Lavrov, 2016).

AGREEMENT WITH REPORTED TRANSLOCATIONS

Our genetic assignments fit well with the reported origin of the newly established populations. The Kirov population, for example, was established after translocations from Voronezh and Belarus (Milishnikov & Saveljev, 2001), which is in perfect agreement with both the new microsatellite results and previously published mtDNA results (Senn *et al.*, 2014). Aside

from the Elbe River population mentioned above, the populations in Czechia were founded through range expansion of highly admixed stock along the Danube and its tributaries in Bavaria and Austria. In addition, beavers were released in eastern Czechia from Lithuania and north-east Poland. The complex origin of these populations is clearly apparent in the microsatellite data, which suggest contributions from French, Belarussian and Norwegian relict populations. Although the Voronezh signal was not well pronounced in the microsatellite data, the Voronezh *JF7* haplotype was present in both western and eastern Czechia. These two Czech populations differ considerably, however, with a strong French signal in western Czechia and absent in eastern Czechia and with the eastern Czech population showing a closer relationship with the Belarussian and Norwegian populations. The present-day population in Bavaria appears to be more complex and admixed (Zahner, 1997; Frosch *et al.*, 2014) than the geographically close western Czech population, suggesting that the genetic structure of the western Czech population might have been established via a founder effect during its early range expansion and that its unique genetic structure is maintained by isolation, owing to the mountain range along the boundary of Bavaria and Czechia. Autochthonous beavers disappeared from Poland and Lithuania before World War II, and the restored population is thought to originate from the Belarus and Voronezh relict populations (Ulevicius & Paulauskas, 2003). Our microsatellite data confirm this supposition, suggesting a mixed Belarus origin, with mitochondrial haplotypes indicating both Belarus and Voronezh as source populations.

CONCLUSIONS

We were able to show that most of Europe, and perhaps Asia, is now populated by beavers with a highly complex origin, the genomes of which often show a high degree of admixture. The joint influence of massive translocations, natural expansion and stochastic effects has created a complicated but strong population structure in Central Europe, where neighbouring populations differ significantly in genetic composition. However, we assume that this pattern is only transient and that populations will become more homogenized as they come into contact in the near future. Natural selection will very probably replace the prevailing influence of stochastic effects in shaping the genetic structure of these beaver populations, opening a new window for future studies using genomic data.

The newly established populations examined in the present study are all viable and expanding rapidly, which suggests that outbreeding depression does not pose a significant threat. On the contrary, it could be

argued that the newly established populations are successful owing to their high genetic diversity, a theory that needs to be investigated in more detail. In addition to genetic ancestry, other factors, such as disease risk and maintaining local adaptations (Girling *et al.*, 2019), should also be considered during future conservation measures. Following from this, we suggest that the distant populations in China, Siberia and Mongolia (*Castor fiber tuvinicus*, *C. f. pohlei* and *C. f. birulai*), which have persisted for a long time in isolation and show clear genetic differentiation, could harbour unique features and, as such, should receive special protection.

We were also able to demonstrate that the ancestry of beavers in newly established populations could be traced back using a limited number of genetic markers. Mitochondrial DNA and microsatellite markers are characterized by a high mutation rate and, as such, are the markers of choice for differentiating recently separated beaver populations. Nonetheless, cross-laboratory comparisons of results based on microsatellite loci are generally limited. As such, there is a need for new methods that provide more exact and transferable scoring of alleles at microsatellite loci, such as sequence-based microsatellite genotyping (Darby *et al.*, 2016; Tibihika *et al.*, 2019).

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the editor, John A. Allen. The authors have no conflicts of interest to declare.

DATA AVAILABILITY

A large part of this study is based on the reanalysis of mitochondrial DNA sequences from previous studies (Table 3; Supporting Information, Table S1). We have not found any new mitochondrial control region haplotype in Czechia populations. The new data comprise microsatellite loci genotypes and are available as Supporting Information (Table S2).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Mitochondrial DNA control region haplotypes of present-day beavers (FASTA format). See [Horn *et al.* \(2014\)](#) and [Marr *et al.* \(2018\)](#) for haplotypes of ancient beavers.

Table S2. Microsatellite loci genotypes (comma-delimited text format).

Table S3. Pairwise F_{ST} values (comma-delimited text format).