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Ancient mitochondrial DNA and the genetic history of Eurasian beaver (*Castor fiber*) in Europe

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Abstract

After centuries of human hunting, the Eurasian beaver *Castor fiber* had disappeared from most of its original range by the end of the 19th century. The surviving relict populations are characterized by both low genetic diversity and strong phylogeographical structure. However, it remains unclear whether these attributes are the result of a human-induced, late Holocene bottleneck or already existed prior to this reduction in range. To investigate genetic diversity in Eurasian beaver populations during the Holocene, we obtained mitochondrial control region DNA sequences from 48 ancient beaver samples and added 152 modern sequences from GenBank. Phylogeographical analyses of the data indicate a differentiation of European beaver populations into three mitochondrial clades. The two main clades occur in western and eastern Europe, respectively, with an early Holocene contact zone in eastern Europe near a present-day contact zone. A divergent and previously unknown clade of beavers from the Danube Basin survived until at least 6000 years ago, but went extinct during the transition to modern times. Finally, we identify a recent decline in effective population size of Eurasian beavers, with a stronger bottleneck signal in the western than in the eastern clade. Our results suggest that the low genetic diversity and the strong phylogeographical structure in recent beavers are artefacts of human hunting-associated population reductions. While beaver populations have been growing rapidly since the late 19th century, genetic diversity within modern beaver populations remains considerably reduced compared to what was present prior to the period of human hunting and habitat reduction.

Keywords: Conservation Biology, Conservation Genetics, Phylogeography, Population Genetics – Empirical

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Introduction

Beavers (genus *Castor*) have the second largest body size of all extant rodents, surpassed only by the capybara, *Hydrochoerus hydrochaeris*. They live along rivers with their main food sources being the bark of trees and soft plants. Thus, beavers are typical representatives of warm-stage fauna, requiring the presence of trees. Beavers were therefore probably absent from central Europe during glacial phases and Holocene beaver populations in central Europe are likely to have been established following postglacial dispersal from refugia. Survival in and postglacial expansion out of southern refugia is not uncommon in warm-adapted Eurasian fauna (Taberlet *et al.* 1998; Hewitt 2000). Although genetic data from some species such as the common vole (Tougard *et al.* 2008) show complex phylogeographical patterns supporting the survival of glacial populations in northern refugia (Stewart & Lister 2001; Stewart *et al.* 2010), given the ecological preferences of beavers, they are more likely to have survived in refugia in southern Europe.

Beavers have long been an important resource for human populations living across the Holarctic, and this will also have influenced the amount of genetic diversity and the extent of phylogeographical structure among modern beavers. Their fur is of exceptional quality and has been a highly traded commodity (Djoshkin & Safonow 1972; Baker & Hill 2003). Beavers have also been hunted for meat, due to their size, and for castoreum – an anal gland secretion often used in traditional medicine. Stone engravings at Lake Onega in northern Europe indicate that beavers played a role in ancient human societies from around 3000–4000 years before present (yBP) (Ravdonikas 1936; Brentjes 1968). Thus, hunting of beavers most likely began thousands of years ago, long before populations of the Eurasian *C. fiber* started to decline dramatically beginning in medieval times (Veron 1992; Halley & Rosell 2003). At the end of the 19th century, a census report indicated that only around 1200 individual beavers survived in Eurasia, and these were distributed among eight, small, relict populations scattered across the region (Nolet & Rosell 1998). In order to maintain the species, conservation management included the protection of existing beaver populations and also the establishment of new populations from various regions of origin (Halley & Rosell 2002). Information on beaver phylogeography and tracking of the populations as they expand is important for conservation management of European populations, for example, to aid the re-introduction of beavers into Great Britain (Macdonald *et al.* 2000; Dolch *et al.* 2002; Heck *et al.* 2009; Horn *et al.* 2010; Halley 2011).

Molecular studies on relict populations of the Eurasian beaver, using mitochondrial (mt) DNA markers, showed that no haplotypes were shared between populations, and described some of the populations as mitochondrially monomorphic (Ducroz *et al.* 2005; Durka *et al.* 2005). Because of similarly low diversity in nuclear major histocompatibility complex (MHC) loci and the known impact of human hunting on Eurasian beaver populations during the previous centuries, it was assumed that the low genetic diversity of this species was caused by a severe population bottleneck due to human hunting (Babik *et al.* 2005). However, as neither the extent of genetic diversity nor the phylogeographical structure of beaver populations prior to human hunting are known, it remains an open question whether the extreme phylogeographical pattern and comparatively low genetic diversity of modern European beaver populations are the result of Pleistocene–Holocene population dynamics, or rather of late Holocene to historic, human-induced population reduction.

Using ancient mitochondrial DNA isolated from 48 beavers ranging in age from several hundred to around 11 000 years old, we investigate (i) how much genetic diversity was lost in beaver populations prior to the 19th century and (ii) whether the extant phylogeographical structure of Eurasian beaver populations is an artefact of its recent, severe population decline or, alternatively, whether strong geographical structure is characteristic of beaver mitochondrial lineages over a much longer time frame. Distinguishing ancient phylogeographical patterns from those superimposed by recent strong drift has implications far beyond resolving the evolutionary history of extant European beaver populations as the issue of drift obscuring population history is likely to also affect other populations of conservation concern.

Materials and methods

Samples and DNA extraction

We attempted to isolate ancient DNA from 119 Holocene *C. fiber* bone and tooth fragments from Central Europe, North Scandinavia and European Russia. Most samples date to the Holocene and few to the late Pleistocene, between several hundred and around 45 000 years before present (yBP). Samples originated from both museum and private collections (Table S1, Supporting Information) and were processed using standard precautions for work with ancient DNA (Pääbo *et al.* 2004; Gilbert *et al.* 2005). Pieces of bone or tooth were powdered manually using mortar and pestle, and DNA was extracted using two published protocols developed specifically for ancient DNA (Rohland &

Hofreiter 2007; Rohland *et al.* 2010). Negative controls were included in both extraction and subsequent enrichment for the mitochondrial control region by PCR and DNA hybridization capture, respectively, to monitor potential contamination.

Screening for endogenous DNA via multiplex PCR

We performed multiplex PCR amplifications (primer sequences listed in Table S2, Supporting Information) to test for the survival of ancient DNA. Extracts that yielded at least one PCR product of the correct length were processed further (see below), to obtain a 495-base pair (bp) fragment of the mitochondrial control region.

Multiplex PCR and sequencing

For 13 samples, the 495-bp mitochondrial control region fragment was amplified in overlapping fragments using multiplex PCR (Römpler *et al.* 2006). Primer sequences used are provided in Table S2 (Supporting Information). PCR products in the multiplex PCR ranged in size from 80 to 120 bp. PCR and subsequent sequencing was conducted at least twice for each locus to determine accurate DNA sequences despite potential ancient DNA damage (Hofreiter *et al.* 2001; Stiller *et al.* 2006; Briggs *et al.* 2007; Brotherton *et al.* 2007). PCR products were sequenced either by Sanger sequencing after cloning or on the 454 platform (Roche). For sequencing PCR products on the 454 platform, the PTS (parallel tagged sequencing) and DMPS (direct multiplex sequencing) tagging protocols were used for barcoding the PCR products prior to sequencing (Meyer *et al.* 2008; Stiller *et al.* 2009). For 75 samples, we used hybridization capture to obtain the required sequences. The remaining 31 samples did not yield sufficient DNA. Detailed information on the protocols used on each sample can be found in Table S1 (Supporting Information).

Generation of bait for DNA hybridization capture

DNA molecules representing the target sequence were generated to serve as 'bait' in hybridization capture. The bait molecules hybridize to the complementary beaver DNA and are then immobilized via attached biotin residues (Noonan *et al.* 2006; Maricic *et al.* 2010; Horn 2012b). To introduce biotin into the bait molecules, we used a low concentration of biotinylated dUTP in the PCR. In detail, a control region PCR amplicon of *C. fiber* ssp. *albicus* was isolated from an agarose gel in order to avoid carryover of genomic template DNA and diluted 1/1000 in double-distilled water. This dilution was then used as template in a PCR with a final concentration of 5 μM biotinylated dUTP, 245 μM dTTP and 250 μM dATP, dGTP and dCTP (Fermentas). After a purification step using a MinElute kit (Qiagen), the bait solution was measured on a Nanodrop™ and used in appropriate concentration (see below) in subsequent hybridization capture reactions.

DNA hybridization capture and sequencing

For 75 ancient DNA samples, we employed DNA hybridization capture to enrich the DNA extracts for the mitochondrial control region. We prepared barcoded genomic libraries for sequencing on either 454 or Illumina GAII sequencing machines using published protocols (Stiller *et al.* 2009; Meyer & Kircher 2010). We amplified sequencing libraries using the Phusion® High Fidelity PCR master mix (Finnzymes) and used these in two serial DNA hybridization capture reactions (Meyer & Kircher 2010). Individual samples were handled in separate tubes and plate wells at all times and were combined only in the final sequencing pool (Horn 2012a).

DNA hybridization capture was carried out for 24 h. Enriched libraries resulting from the first hybridization were captured via streptavidin-coated magnetic beads, amplified using the Phusion® High Fidelity PCR master mix, cleaned up using a MinElute column (Qiagen) and used in the second hybridization. For 454 libraries, around 500 ng of library was incubated with around 50 ng of bait in both hybridization steps. Hybridizations were carried out in different tubes for each library (Eppendorf), rotating at 65 °C in a conventional hybridization oven (SciGene). Moving from tubes to a plate format, the amount of library and bait used in the hybridizations was reduced, but a ratio of 1:10 for the amount of bait to library DNA was retained. Thus, for Illumina libraries, around 170 ng of library was incubated with around 17 ng of bait in both hybridizations. Hybridization capture of each library was carried out in a different well of one 96-well plate in a conventional thermocycler without rotation.

After the second round of capture, the resulting enriched libraries were either quantified, pooled and sequenced (454 libraries) or amplified once more before quantification, pooling and sequencing (Illumina libraries). The respective combinations of library preparation and sequencing method are listed in Table S1 (Supporting Information) for each sample. Negative controls were incorporated throughout DNA extraction, preparation of sequencing libraries, target enrichment and quantitation. In gel electrophoresis of amplified libraries, negative controls consistently only showed products with the fragment length of adapter dimers instead of the length expected for PCR products plus adapters as well as low copy numbers around

$1–3 \times 10^5$ copies per microlitre (cp/μL) in qPCR, whereas most samples yielded between 3×10^6 and 1×10^8 cp/μL (Horn 2012a).

Sequence analyses

A reference sequence of a *C. fiber* control region was created as a consensus sequence from the previously published modern *C. fiber* sequences by majority rule. Sequence reads from 454 runs were sorted for their barcodes using the software untag (https://bioinf.eva.mpg.de/pts/; Meyer *et al.* 2008) and mapped onto the reference sequence using runMapping (454 Roche software). To control for misincorporations induced by miscoding lesions when capturing ancient DNA by hybridization, we required multiple unique molecules to call a consensus base. Unlike in PCR, unique molecules can be easily identified by their start and end positions in an alignment so that each molecule with different start and/or end coordinates can be considered a unique template molecule, whereas molecules with identical start and end coordinates are likely to be duplicates of the same original template molecule that result from library amplification. After collapsing those duplicates, we required a minimum coverage of three unique reads for each position in order to call a consensus sequence as with Sanger-sequenced PCR replicates (Meyer & Kircher 2010; Horn 2012b).

Illumina base calling was performed using IBIS (Kircher *et al.* 2009), and the index sequences were used to assign reads to samples (Meyer & Kircher 2010). One sample was discarded because of a read count below 10 000 reads. We estimated the false assignment rate of indices by evaluating the number of reads showing an index that had not been used, resulting in 1 in 6400 reads falsely assigned. This value is in the range of previously reported false assignment rates of between 1 in 1000 and 1 in 10 000 (Meyer *et al.* 2008). Sequencing reads were mapped against the control region sequence using BWA version 0.5.5 (Li & Durbin 2009), requiring a minimum mapping quality of 20 and a minimum fragment length of 30 bp. For the beaver samples, on average around 24% of the reads could be mapped. Negative controls and the unused index yielded only around 5% of the reads mapped. Therefore, samples with less than 5% of the reads mapping to the reference sequence were discarded at this stage. Because the experiment included several amplifications of the libraries, only high-frequency reads (observed at least 10 times) were accepted as truly amplified molecules, whereas reads observed less than 10 times were discarded (Horn 2012a). Samples with less than 20 high-frequency reads spanning the ~500-bp target were also discarded. For two beaver samples, we used additional PCR-derived sequences to fill gaps in the target sequence. Finally, only target sequences that were covered with at least three sequencing reads over 95% of their length or more were accepted. The bam files with sequencing reads are available upon request. Eight of the 48 ancient beaver sequences were previously published as part of a book chapter describing the ancient DNA hybridization capture protocol (GenBank Accession nos. HQ880649-HQ880656, see Table S1 (Supporting Information) (Horn 2012a)).

Phylogenetic analyses and genetic diversity

The *C. fiber* control region sequences obtained in this study were combined with published sequences of recent *C. fiber* (GenBank Accession nos. AY623632–43 and DQ088700–03, (Durka *et al.* 2005)) and aligned using MAFFT version 6.708b (Katoh *et al.* 2005). The full-sequence data set contained 152 modern and 48 ancient individuals. The alignment was trimmed to 495 bp (including gaps), the length that was available for the published modern sequences (Supporting Information). We constructed a median joining network with NETWORK version 4.5.1 (Fluxus technology Ltd), using weighted distance for missing data in pairwise comparisons (Bandelt *et al.* 1999). Haplotype diversity, nucleotide diversity and Fst values were calculated in DnaSP (Rozas *et al.* 2003), excluding sites with alignment gaps and ambiguities only in pairwise comparisons (Table S3, Supporting Information). Standard deviations are reported as calculated by DnaSP and diversity estimates including heterochroneous DNA sequences were corrected according to (Depaulis *et al.* 2009) equation (2).

To investigate divergence times of different clades and population dynamics over time, we inferred genealogies using the strict molecular clock model as implemented in BEAST version 1.7.5 (Drummond & Rambaut 2007). The number of ancient sequences was therefore restricted to 42 that had sufficient age information (Table S1, Supporting Information). Initially, we attempted to calibrate the molecular clock rate by fixing the age of the branches leading to ancient sample to the sample age in years before present (age estimates for ancient samples are based on the context of the respective site given by the zooarchaeologists and on ^{14}C dating for one sample; Table S1, Supporting Information). However, comparison to a null model (running an identical BEAST analysis but without sequence data) indicated insufficient information to calibrate the clock via this approach. No reasonable estimate for the molecular rate of the mitochondrial hypervariable region for beavers was available in the published literature. However, a previous analysis of the complete mitochondrial genomes of a variety of rodent species suggested that

beavers have a relatively slow rate of molecular evolution, on the order of 1.0×10^{-7}–1.5×10^{-7} mutations/site/year. Because this rate is both fossil-calibrated and estimated for the entire mitochondrial genome, it is likely to be an extreme lower bound of the evolutionary rate for this portion of the mitochondrial genome. To span the range of reasonable rates estimated from similar rapidly evolving regions of the mitochondria in other ancient DNA studies (Lambert *et al.* 2002; Shapiro *et al.* 2004; Lorenzen *et al.* 2011), we performed four separate analyses in which, in addition to fixing the ages of these ancient tips, we fixed the clock rate to either 1.0×10^{-7}, 3.0×10^{-7}, 5.0×10^{-7} or 7.0×10^{-7} substitutions/site/year.

The median joining network identified three distinct clades comprising individuals from three geographically distinct regions, indicating strong population structure among historic beavers. We therefore performed BEAST analyses as described above on three distinct data sets: (i) the full data set that included individuals from all three clades identified in the median joining network; (ii) the western clade only; and (iii) the eastern clade only. The latter two analyses more accurately reflect distinct geographical 'populations' and are therefore more appropriate for analyses that assume a coalescent process. We did not perform a separate analysis on the Danube population because too few individuals are sampled to obtain precise demographic estimates.

In addition to the molecular clock parameters as described above, we assumed the HKY+G model of nucleotide substitution, as suggested by jModelTest (Posada 2008). For all three data sets, we assumed two different coalescent priors: a constant population size and the flexible Bayesian skygrid model (Gill *et al.* 2013). For each of the 24 BEAST analyses, we ran two independent MCMC chains for 60 million iterations each, sampling trees and model parameters from the posterior every 6000 iterations. The first 10% of each run were discarded as burn-in and, after visual inspection for sufficient sampling and chain convergence using TRACER version 1.5 (Rambaut & Drummond 2012), the remainder combined. Maximum clade credibility (MCC) trees were generated using TREEANNOTATOR version 1.7, which is distributed as part of the BEAST software package. Skygrid plots were generated using a custom R script provided by M. Suchard. For each data set, we compared marginal likelihoods of trees resulting from the constant and skygrid coalescent models using both the stepping stone (SS) and path sampling (PS) protocols (Beale & Lennon 2012). In all cases, the constant population size model was rejected in favour of the more complex skygrid model.

To accommodate potential oversampling of the modern beaver populations, we also reduced the number of modern sequences in the data set to 47 representing the modern haplotypes according to their respective frequencies in the modern populations (Durka *et al.* 2005). Thus, the smaller set mirrored the haplotype frequencies of the larger set. BEAST runs on this 'downsampled' data set yielded similar results.

We used approximate Bayesian computation (ABC; Beaumont *et al.* 2002) analyses to quantitatively test different population scenarios. ABC approximates posterior distributions of model parameters using information from prior distributions and extensive simulations rather than calculating the likelihood of the data directly. Bayesian Serial Simcoal (Anderson *et al.* 2005) was used to simulate the temporal DNA data (each clade separately) under a closed one population constant size, a closed one population increase bottleneck and a closed one population bottleneck model (Table S4, Supporting Information). We excluded the third phylogenetic clade from the Danube Basin from the ABC analyses, due to its small sample size. We ran Bayesian Serial Simcoal with 1 000 000 iterations for each of the models. The number of segregating sites, the number of haplotypes and nucleotide diversity served as summary statistics for the 2 (east) and 3 (west) averaged time levels, respectively. In total, this resulted in 6 (east) and 9 (west) summary statistics. We applied a nonlinear regression-based machine learning approach (Blum & François 2011), which is implemented in the freely available 'ABC' R package (Csilléry *et al.* 2012). We used tolerance levels of 0.001, 0.002 and 0.004 for the ABC parameter estimations (Table S5, Supporting Information). We further used expected deviance (deviance information criterion (DIC); implemented in the R package 'ABC') to infer the best supported model (Table S6, Supporting Information). To do so, we simulated the respective models with 1000 parameters sampled from the posterior distribution as fixed model parameters. The corresponding summary statistics were then used to calculate the DIC values.

Results

Utilizing multiplex PCR and DNA hybridization capture, we were able to sequence a 495-bp fragment of the mtDNA control region for 48 of 119 ancient beaver specimens, resulting in a success rate of ~40%. In total, we found 43 haplotypes in the 48 ancient individuals sequenced. All haplotypes were different from the 16 modern haplotypes found in an earlier study (Durka *et al.* 2005). The ancient beaver sequences combined with the sequences obtained for modern beavers provide information on the distribution of mitochondrial

lineages of European beaver populations before and after human impact.

Phylogeography and migration routes

A median joining network (Fig. 1A) and Bayesian genealogical analysis (Fig. 1B) of the sampled mitochondrial sequences both reveal three major clades and one well-supported subclade. The pattern of divergence among the three major clades could not be resolved with statistical support using our data. The phylogenetic results suggest strong phylogeographical subdivision among ancient beavers. Sequences from the two major clades originate from western and eastern Europe, respectively, while the third, smallest clade consists exclusively of sequences originating from the Danube Basin (Fig. 2). All sequences in this third clade are from fossil samples, as beavers went extinct in Romania in 1824 (Halley & Rosell 2003). Thus, the group of ancient samples from the Danube Basin represents a newly identified, genetically distinct mitochondrial lineage that appears to have disappeared during the 19th century. Fst values for the ancient samples are 0.61 (east–west) and 0.73 (east or west – Danube; Table 1), also supporting substantial genetic structuring among the three clades.

Except for the separation of eastern and western European beavers and a well-supported subclade within the western clade comprising sequences from southern Scandinavia, there is little evidence for ancient phylogeographical structure on the European mainland. Thus, the extreme phylogeographical structure present among living beaver populations, with each population carrying private alleles (Durka *et al.* 2005), is likely to be a consequence of recent population decline (Djoshkin & Safonow 1972; Nolet & Rosell 1998). The ancient data also reveal a potential contact zone between the western and eastern clades in eastern Europe. In one location (near Gluchowo, Poland), two western clade beavers were present at around 2850 yBP and two eastern clade beavers were present at around 850 yBP (sequence IDs POL1-4, Table S1, Supporting Information). As these dates are separated by ~2000 years, it is, however, also possible that the two clades never overlapped; additional sampling will be required to further test this hypothesis.

We were not able to extract DNA from ancient samples from any of the proposed European Pleistocene refugia and are therefore unable to address the broad, postglacial recolonization history of beavers in Europe. Our data do show, however, that beavers colonized Scandinavia from both western and eastern Europe, with beavers from southern Scandinavia falling within a subgroup of the western clade and those from northern Scandinavia into the eastern clade (Fig. 1).

Genetic diversity

By including ancient mitochondrial DNA sequences, our data also allow insights into past population dynamics of European beavers. While extant beaver populations harbour very little genetic diversity (16 haplotypes in 152 individuals), the ancient samples show a much greater number of mitochondrial haplotypes, with 43 haplotypes isolated from 48 samples (Fig. 3). The loss of genetic diversity from the past to the present affected all three inferred clades: the Danube clade was lost entirely, the western clade was reduced from 27 to 4 haplotypes, and in the eastern clade, 12 haplotypes were identified among 91 modern samples, and nine haplotypes among ten ancient samples (Fig. 3). It is also noteworthy that, within the current sampling, not a single haplotype is shared between the modern and the ancient data set (Fig. 3). Haplotype diversity decreased from 0.99 in ancient to 0.88 in modern beavers (Table 1 and S3, Supporting Information). In the western clade, the haplotype diversity decreased from 0.98 to 0.68 and in the eastern clade from 0.99 to 0.81.

To test the hypothesis of a recent population decline, we inferred changes in female effective population size separately for western and eastern clade beavers using the skygrid plot (Gill *et al.* 2013) as implemented in BEAST version 1.8 (Drummond *et al.* 2012). We used two fixed molecular clock rates: 1.0×10^{-7} substitutions/site/year, which reflects the extreme lower range of possible rates for this region of the mitochondrial genome for Eurasian beavers (Horn *et al.* 2011), and 5.0×10^{-7} substitutions/site/year, which reflects an average (fast) rate of those estimated from other ancient DNA data sets (Fig. 4). Regardless of the molecular rate assumed, the plots show decreasing effective population size in both clades, with a more pronounced pattern of decline in the western clade, beginning around the transition into the Holocene.

To further explore genetic support for past and present genetic structure among beaver populations, we performed simulations of several demographic scenarios using approximate Bayesian computation (ABC). The parameter estimations for the respective best supported model can be found in Table S5 (Supporting Information). We inferred constant population size as the best supported model for both the western and the eastern clade (Table S6, Supporting Information) and estimated female effective population sizes to be small for both populations [Table S6, Supporting Information, median: 972 (median; west) and 2317 (median; east)]. In contrast to our findings from the skygrid analyses, ABC did not support a decreasing population size. However, we have to note that the current sampling is not ideal

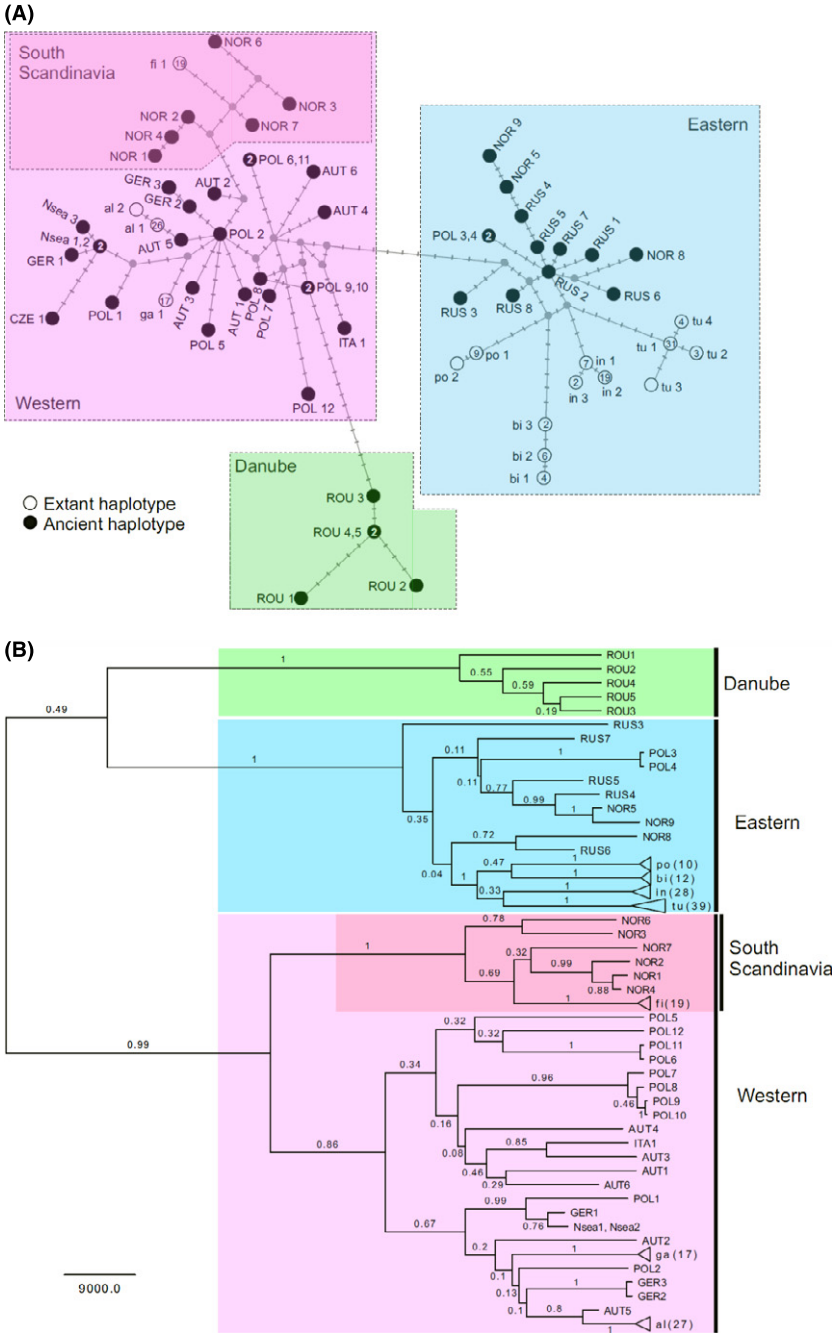

Fig. 1 Phylogenetic relationships among ancient and extant *C. fiber*. (A) Median joining network of 495 bp of ancient (black), extant (white) and hypothetical (grey) mitochondrial haplotypes. Each step on the lines represents one mutation difference between the connected haplotypes. More information about each specimen can be found in Table S1 (Supporting Information). (B) Maximum clade credibility (MCC) tree with Bayesian posterior support values provided at the nodes. The genealogy was generated using BEAST with the dated ancient and 152 modern sequences. Results of the analysis using a fixed rate of 5.0×10^{-7} substitutions/site/year are depicted; however, no significant topological differences were observed in analyses using different rates. Clades of identical sequences in modern beavers have been collapsed, with the number of individuals indicated in parentheses. The three major clades and one minor (southern Scandinavia) clade identified in the median joining network are all highly supported in the Bayesian phylogenetic analysis.

for ABC analyses for two reasons: (i) analyses have shown that the best results are obtained for data sets around and above 20 samples per time average (Ramakrishnan *et al*. 2005) and we only have 13 ancient samples for the eastern population and 11 for the oldest ancient time average in the western population; (ii)

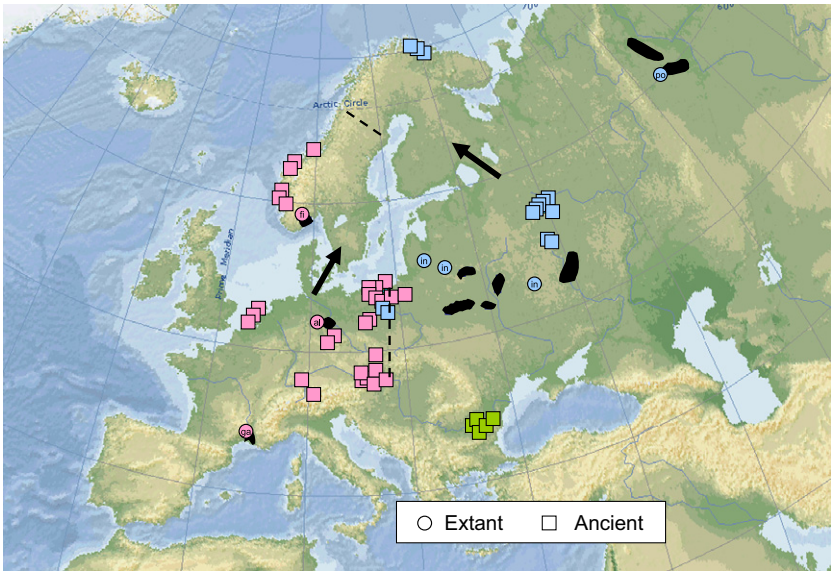

Fig. 2 Map of ancient and extant *C. fiber* and dispersal routes in Europe. The western clade (pink) dispersed northwards and colonized southern Scandinavia, while the eastern clade (blue) dispersed into Scandinavia via a northern route (arrows). Sampling sites are indicated for ancient (squares) and recent (circles) mtDNA haplotypes. Dotted lines indicate the position of Holocene contact zones found for other species (Hewitt 2004). A divergent population existed in the Danube Basin (green) but is extinct today. Black areas indicate *C. fiber* relict populations that survived to the present day. Recent mtDNA haplotypes are indicated near to the relict populations and represent different subspecies (al: *C. f. albicus*, ga: *C. f. galliae*, fi: *C. f. fiber*, in: *C. f. belorussicus*/orientoeuropaeus, po: *C. f. pohlei*).

Table 1 Measurements of genetic diversity of *C. fiber* during the Holocene. Haplotype diversity h, nucleotide diversity π and Fst values were obtained using DnaSP. Details including standard deviations can be found in Table S3 (Supporting Information). Note that the nucleotide diversity estimate for the ancient population sample contains heterochroneous sequences and was corrected using (Depaulis *et al.* 2009) equation (2)

Measurement	Ancient	Extant
Individuals sampled	48	152
Haplotypes	43	16
Haplotype diversity (h)	0.99	0.88
Nucleotide diversity (π)	0.0205	0.0291
Fst east–west	0.61	0.56
Fst east–Danube	0.73	—
Fst west–Danube	0.73	—

because we only have one ancient time average for the eastern population, the inferred bottleneck timing likely falls within our ancient sample group, which thus contains both pre- and postbottleneck statistics. This likely reduces the power and accuracy of our ABC analysis, which could be the reason why, in contrast to the Skygrid analysis, models of demographic change were not supported over constant size models. Finally, the DIC values show that none of the models was substantially better supported than all others (Table S6, Supporting Information), suggesting that the data set does not contain sufficient information to distinguish among different demographic models with ABC.

Discussion

Recent population decline and implications for taxonomy

Holocene climate changes have been implicated in the decline of genetic diversity in a number of species, including North American caribou (Kuhn *et al.* 2010) and lemmings in Eurasia (Prost *et al.* 2013) and North America (Fulton *et al.* 2013). However, these species are all cold-adapted and thus prefer the colder climate of the Late Pleistocene over the warmer Holocene, which may explain the relationship between losses in genetic diversity and climate. Interestingly, species that are better adapted to the warmer conditions of the Holocene have also experienced considerable declines within the last 10 000 years. These declines are often blamed on widespread encroachment by growing human populations through, for example, destruction and fragmentation of habitat and/or increased hunting pressure. In Europe, for example, hunting by humans is thought to have contributed to the decline of European brown bears (Valdiosera *et al.* 2008) and shifts in genetic diversity in European rabbits (Hardy *et al.* 1995). Populations

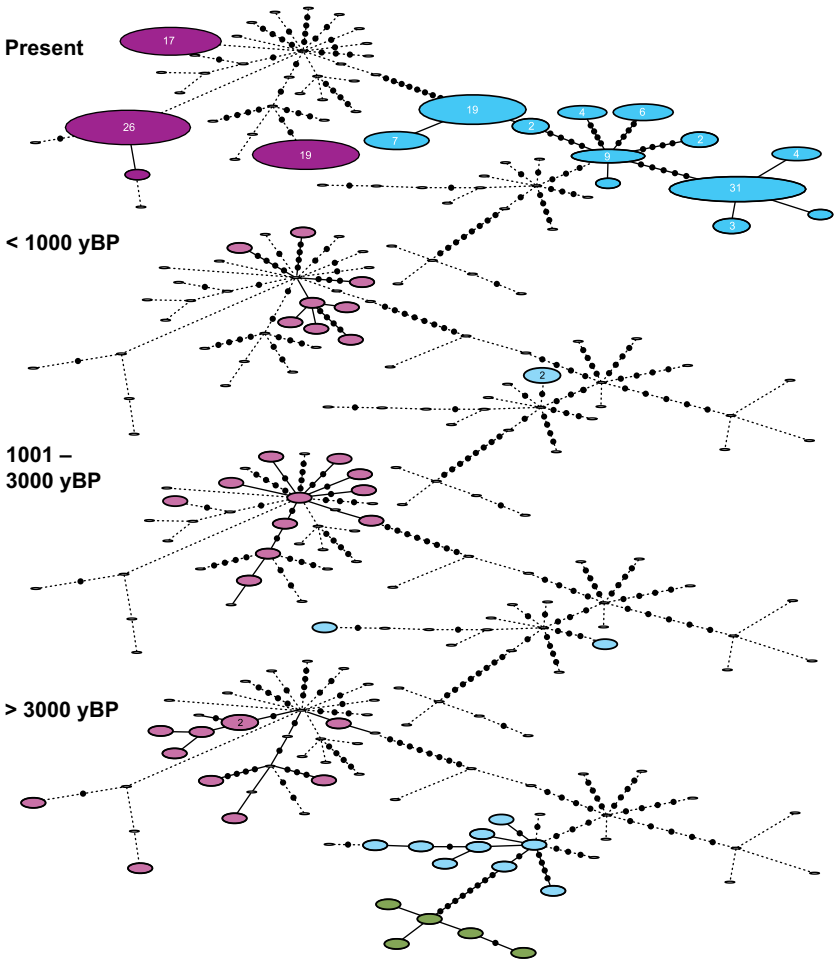

Fig. 3 Temporal haplotype network reconstruction. Haplotypes are represented by ellipses. The colours correspond to the three clades: west (pink/purple), east (blue) and Danube (green). The three bottom layers represent ancient samples, and the top layer depicts extant samples. Age ranges for the individual layers are given on the left side. The number of sequences sharing the same haplotype is given inside the respective ellipse, with only numbers bigger than 1 shown. Two haplotypes are connected by a line if they are separated by one substitution; each additional substitution is indicated by a small black dot. Small white ellipses correspond to haplotypes found in the entire sampling, but not the particular time layer. These small white ellipses are connected by dotted lines.

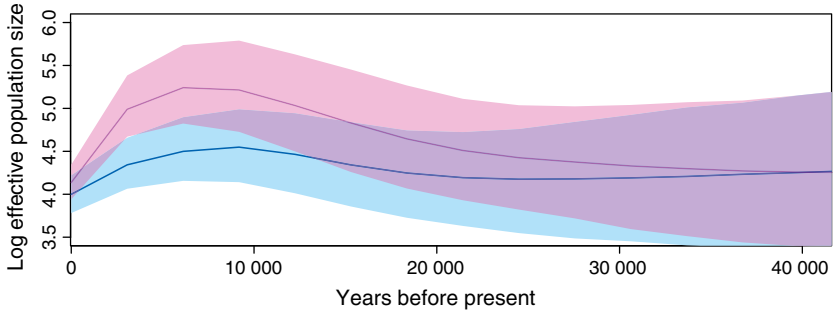

Fig. 4 Bayesian skygrid plots of female effective population size through time for the western (pink) and eastern (blue) beaver clades. Results of the analyses assuming a strict clock rate of 5.0×10^{-7} substitutions/site/year are shown. These analyses indicate similar timings of the initial population declines. The *y*-axis shows theta, an approximation of female effective population size under the assumption of no population substructure. Both plots show a decrease in female effective population size towards the present day.

of European brown bears have also gone locally extinct during this interval: a mitochondrial clade that had survived to the Holocene in southern France disappeared around 5000 years ago, presumably a consequence of human encroachment (Valdiosera *et al.* 2007, 2008).

Beavers are warm-adapted fauna that should thrive in the warmer conditions of the Holocene, as forests expand and more habitat for beavers becomes available. However, our data suggest that, rather than an increase in genetic diversity following postglacial expansion,

beavers lost diversity across Eurasia during the Holocene, with particularly severe declines in several newly colonized locations. Our data, in combination with documented, more recent population declines, suggest that European beavers are another example of a species that has been strongly affected by expanding human populations during the Holocene. Indeed, most of the recent demographic history of Eurasian beavers is strongly linked to humans (Halley & Rosell 2002, 2003; Dewas *et al.* 2012).

Some authors have argued that the recent demography of Eurasian beavers resembles a near-extinction event that can be blamed on human overexploitation (Linstow 1908; Veron 1992; Durka *et al.* 2005). Our data provide some support for this hypothesis, in particular when considering each beaver population separately. Extinction is ultimately a demographic process. While genetic diversity is predicted to be low in a population immediately prior to extinction, the process of reduction in genetic diversity depends on many factors, including population structure and the rate of population decline. Our results show that *C. fiber* harboured more mitochondrial diversity in the past and that the extant population as a whole retains some genetic diversity. However, key to understanding the recent demographic history in beavers is to consider each of the three populations (represented by the three distinct clades) separately. While the eastern population only shows a moderate decline in genetic diversity, the other two populations show much more dramatic losses. The Danube population went extinct, and although multiple haplotypes survived in the western population, they only survived due to lineage sorting among the various relict populations. Therefore, while overall beavers do not seem to have nearly gone extinct, the loss of habitat and resulting fragmentation into relic populations, each with only a few or even single surviving mitochondrial haplotypes, do support a hypothesis of local near-extinctions.

According to historical records, the most rapid period of decline in *C. fiber* populations continued until late in the 19th century, when conservation statutes were put into place to encourage the recovery of the Eurasian beaver populations (Nolet 1997; Halley & Rosell 2002). This rapid loss of diversity prior to conservation efforts appears to have established a much stronger phylogeographical structure among present-day beaver populations than that which existed during the early Holocene. Although our ancient DNA sequences show phylogeographical differentiation among ancient beaver populations (Fig. 1), we distinguish only three major clades, with little phylogeographical structure within the two larger clades. In contrast, previous analyses of modern beavers have suggested much stronger phylogeographical structure among Eurasian beavers, even going so far as to propose eight distinct subspecific taxonomic units to define the geographical populations (Babik *et al.* 2005; Ducroz *et al.* 2005; Durka *et al.* 2005).

Our data suggest that this classification is an artefact of recent population decline, rather than the consequence of long-term genetic isolation. For example, in our analysis, the haplotypes from the extant relict populations from Germany and France (currently described as subspecies *C. f. albicus* and *galliae*, respectively) fall into one group, together with ancient beavers from the North Sea, Austria and Poland, and do not show substantial amounts of differentiation either from these or from each other. This suggests that the relict populations from Germany and France are descendants of the same western European beaver population and therefore do not warrant separate subspecies assignment. In conservation management, evolutionary significant units (ESUs) may be of value for distinguishing groups of beavers to be managed independently, if this is desired (Moritz 1994). The two ESUs of western and eastern beavers, established previously (Durka *et al.* 2005), are supported by our data and may be considered in the future management of beavers. Using ancient DNA analyses, beaver mtDNA haplotypes could also be determined for historical, now extinct, populations. For the planned re-introduction of beavers in Great Britain, such data would be useful. However, we could not obtain British specimens to determine ancient mtDNA haplotypes for this region. We also note that our conclusions are based strictly on a survey of short mitochondrial DNA haplotypes and that further genetic work, in particular work involving nuclear DNA, may reveal different evolutionary histories than that inferred from the mitochondrion.

Pleistocene refugia and postglacial dispersal

During the Pleistocene, the Eurasian beaver's habitat spanned a vast region from western Europe to eastern Siberia (Linstow 1908). In southern Eurasia, beaver fossils are known from the Pleistocene of Italy, Spain, Turkey, Syria, Iraq and Iran, all areas where the species was already extinct by the late Holocene (Linstow 1908; Legge & Rowley-Conwy 1986; Barisone *et al.* 2006). For some of these regions, it remains unclear whether beavers persisted throughout the last glacial maximum or recolonized the area after the transition to the Holocene, and later became locally extinct.

The phylogeographical pattern of prebottleneck beavers in Europe resembles the pattern of several other extant species, described by Hewitt as the 'bear-pattern' (Hewitt 2000), with a major western, a major eastern and a minor southeastern clade. Although the

Pleistocene refugia for the western and eastern clades cannot be determined with the data that are currently available, given the ecological preferences of beavers, as in other warm-stage species, the western refugium most likely was on the Iberian Peninsula (Taberlet *et al.* 1998; Hewitt 2000). Moreover, the data allow investigating the postglacial dispersal of beavers into Scandinavia. The oldest western clade beaver found in Scandinavia dates to ~5000 yBP, and the oldest eastern clade beaver is found in northern Scandinavia around ~6200 yBP (Table S1, Supporting Information, Fig. 2). These dates are younger than the timing of deglaciation of the Scandinavian ice sheet around 12500 yBP (Rinterknecht *et al.* 2006) and suggest that Scandinavia may have been colonized via two different dispersal routes. Similar dispersal routes and timing of colonization have been suggested for other forest species, including the brown bear *Ursus arctos* (Taberlet *et al.* 1995) and the badger *Meles meles* (Marmi *et al.* 2006). After their initial colonization of Scandinavia, the sea level rise of the Baltic Sea may have disrupted any dispersal routes connecting southern Scandinavian and central Europe, thereby establishing a distinct southern Scandinavian beaver clade.

In central Europe, the data indicate a strong west–east differentiation among beaver mitochondrial haplotypes. However, members of both clades were observed at sites in Poland that are geographically proximate. This suggests an ancient contact zone in a location that is similar to contact or hybridization zones proposed for other mammals, such as *Mus musculus*, *Ursus arctos* and *Erinaceus europaeus* (Hewitt 2004; Teeter *et al.* 2008; dashed lines in Fig. 2). Today, a contact zone between the major extant clades of *C. fiber* is observed in the area of the Oder River, close to the putative ancient contact zone (Dolch *et al.* 2002; Horn *et al.* 2010). The present-day contact zone was assisted by conservation management and is located to the west of the ancient sites in Poland. However, members of the same phylogenetic clades (western and eastern beavers) meet there today, as they did in the past, suggesting that conservation management may, in the long run, help to restore the populations of threatened species prior to human impact.

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S.H. and M.H. designed the study; D.M., T.K., N.B., E.P., A.K.H. and C.S. provided samples and served as scientific advisors; S.H. generated sequence data; S.H., S.P., M.S. and B.S. analysed the data and S.H., B.S. and M.H. wrote the manuscript.

Data accessibility

DNA sequences: EMBL Accession nos. HG915912-HG915951. Sampling details and haplotype alignments: supplemental online information.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Sampling details.

Table S2 Primers for ancient DNA single- and multiplex PCR amplifications.

Table S3 Measurements of genetic diversity during the Holocene obtained in DnaSP.

Table S4 ABC prior distributions.

Table S5 Estimated ABC parameters for the respective best supported model for the western and eastern populations.

Table S6 ABC model selection using expected deviance (DIC).