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Mitochondrial phylogeography of the Eurasian beaver *Castor fiber* L.

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Abstract

Nucleotide variation in an approximately 490 bp fragment of the mitochondrial DNA control region (mtDNA CR) was used to describe the genetic variation and phylogeographical pattern in the Eurasian beaver (*Castor fiber*) over its entire range. The sampling effort was focused on the relict populations that survived a drastic population bottleneck, caused by overhunting, at the end of the 19th century. A total of 152 individuals grouped into eight populations representing all currently recognized subspecies were studied. Sixteen haplotypes were detected, none of them shared among populations. Intrapopulation sequence variation was very low, most likely a result of the severe bottleneck. Extreme genetic structure could result from human-mediated extinction of intermediate populations, but it could also be an effect of prior substantial structuring of the beaver populations with watersheds of major Eurasian rivers acting as barriers to gene flow. Phylogenetic analysis revealed the presence of two mtDNA lineages: eastern (Poland, Lithuania, Russia and Mongolia) and western (Germany, Norway and France), the former comprising more divergent haplotypes. The low level of sequence divergence of the entire cytochrome *b* gene among six individuals representing six subspecies suggests differentiation during the last glacial period and existence of multiple glacial refugia. At least two evolutionary significant units (ESU) can be identified, the western and the eastern haplogroup. The individual relict populations should be regarded as management units, the eastern subspecies possibly also as ESUs. Guidelines for future translocations and reintroductions are proposed.

Keywords: bottleneck, *Castor fiber*, conservation, control region, mitochondrial DNA, phylogeography

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Introduction

Pleistocene glaciations dramatically affected the biota of the Northern Hemisphere (Hewitt 2000, 2004). Climatic changes during this period triggered range shifts, isolation in glacial refugia, local extinctions, recolonization events, repeated bouts of secondary contact, and severe demographic oscillations (Hewitt 1999, 2000, 2004; Avise 2000). Such historical events left their traces in the levels of intraspecific genetic variation and its geographical distribution. Phylogeography utilizes these present patterns of genetic variation to infer the historical changes in species distribution and abundance (Avise 2000). In Europe, a comparative phylogeographical approach has been particularly successful in unravelling both common patterns and the differences in responses of individual species to climatic and environmental change (Bilton *et al*. 1998; Taberlet *et al*. 1998; Petit *et al*. 2003; Hewitt

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2004). So far, the majority of pertinent analyses have focused on western Europe, the Mediterranean region and Scandinavia. Detailed phylogeographical studies of species with broad Eurasiatic distributions have been relatively uncommon (e.g. Durand *et al*. 1999; Jaarola & Searle 2002; Brunhoff *et al*. 2003; Flagstad & Roed 2003; Babik *et al*. 2004; Deffontaine *et al*. 2005). However, eastern Europe, the Urals and western Siberia were postulated as major refugial areas and are likely to harbour substantial genetic diversity, including lineages of the uttermost importance for conservation (Taberlet & Bouvet 1994; Bilton *et al*. 1998; Taberlet *et al*. 1998; Jaarola & Searle 2002; Babik *et al*. 2004). Therefore, phylogeographical analyses of entire Eurasiatic distributions of widespread species are of high priority. Here we present a range-wide survey of a formerly widespread species that has recently undergone a severe bottleneck of anthropogenic origin. Thus, in indigenous populations, the effects of bottlenecks and range fragmentation are likely superimposed on the genetic legacy of the Pleistocene glaciations and postglacial expansion.

The Eurasian beaver *Castor fiber* L., 1758, is characterized by a remarkable and dramatic demographic history. In early historical times this species was widely distributed in the deciduous and coniferous forest zones of Eurasia, its range extending from western Europe to eastern Siberia (Djoshkin & Safonow 1972), and constituted a key element of the Palaearctic fauna. However, habitat degradation and especially overhunting for fur and chemical substances (castoreum) have led to the extirpation of *C. fiber* from much of its historical range, and to drastic reduction of population sizes (Veron 1992; Nolet & Rosell 1998). At the end of the 19th century the species was on the verge of extinction, surviving only in eight isolated regions: in the Rhone Delta (France), on the middle Elbe (Germany), in south Norway (Telemark), in the Dnepr River system with Beresina and Pripjat (Belarus and the Ukraine), in the Woronesh-Don-system (Russia), in the Konda and Soswa rivers (East Ural region, Russia), on the upper Jenissej and Azas River (Russia, Tuva Republik) and in the Bulgan River (Mongolia, China) (Nolet & Rosell 1998; Fig. 1). In Turkey, the beaver might have survived until the 20th century; however, it is most likely extinct today (Kumerloeve 1975). Total population size of *C. fiber* at the end of the 19th century is estimated as *c*. 1200 animals (Nolet & Rosell 1998). Fortunately, a series of protection and management measures ensured the survival of *C. fiber* in the eight regions. Furthermore, translocations and reintroductions have led to the re-establishment of the beaver over a large part of its former range. Current population size is estimated at 639 000 (Halley & Rosell 2002, 2003), and although a number of reintroductions were not particularly successful, many beaver populations show high viability and the species is rapidly expanding.

A subspecific rank is commonly ascribed to each relict population, supported by skull morphometric analyses (Freye 1960; Lavrov 1979; Heidecke 1986; Frahnert 2000). The Eurasian beaver would thus be divided into eight subspecies: *C. f. albicus* Matschie (1907), *C. f. belorussicus* Lavrov (1981), *C. f. birulai* Serebrennikov (1929), *C. f. fiber* L., 1758, *C. f. galliae* Geoffroy, 1803, *C. f. orientoeuropaeus* Lavrov (1981), *C. f. pohlei* Serebrennikov (1929) and *C. f. tuvinicus* Lavrov (1969) (Serebrennikov 1929; Lavrov 1981; Fig. 1). The taxonomy and systematics of the two relict populations of the eastern European beaver is not settled because three names are available (*C. f. belorussicus*, *C. f. orientoeuropaeus* and *C. f. vistulanus* Matschie, 1907) for one to three potential taxa; in case only one taxon is valid, *C. f. vistulanus* may apply (Gabrys & Wazna 2003). A view that two western European forms (*C. f. albicus* and *C. f. galliae*) represent the separate species *C. albicus* (Lavrov 1979) is not widely accepted.

Many introductions and translocations during the beaver reintroduction programmes were conducted with animals of various geographical origins resulting in mixed populations. The Danube River system, where beavers from Sweden, Poland, Russia and France were released in Bavaria and Austria is an example of such an admixture (Schwab & Lutschinger 2001). Furthermore, this mixed population has been used as source population for reintroductions in several countries (Schwab & Schmidbauer 2003). The situation is complex in the eastern European beavers that spread in the eastern part of the range. Here, in addition to the taxonomical and nomenclatural problems, extensive translocations and reintroductions of animals of diverse geographical origin in some parts of Russia (e.g. Milishnikov & Saveljev 2001) likely led to the amalgamation of two east European forms *C. f. belorussicus* and *C. f. orientoeuropaeus*.

Furthermore, *Castor canadensis* was introduced in certain areas to sustain beaver populations. As a consequence, native Eurasian beavers have now been entirely displaced from certain regions in Finland and Karelia (Fig. 1, Halley & Rosell 2002). However, no successful hybridization between *C. canadensis* and *C. fiber* has been documented (Djoshkin & Safonow 1972; Kuehn *et al*. 2000), and given their different karyotypes (Lavrov & Orlov 1973; Zernahle & Heidecke 1979; Ward *et al*. 1991) introgression of *C. canadensis* alleles into the Eurasian beaver populations should be impossible. No introductions of either species were reported in the areas where other relict populations have survived (the Rhone and Elbe River basins, southern Norway, western Siberia, southern Siberia and central Asia), so apparently the current genetic diversity in these regions reflects the original gene pool.

Studies on genetic differentiation within *C. fiber* are limited, geographically restricted and often led to conflicting conclusions regarding the level of genetic variation and differentiation within *C. fiber* (Ellegren *et al*. 1993; Milishnikov *et al*. 1994, 1997; Kohler *et al*. 2000; Milishnikov & Saveljev 2001). Ellegren *et al*. (1993) observed almost no genetic variation in Scandinavian beavers using data from minisatellites

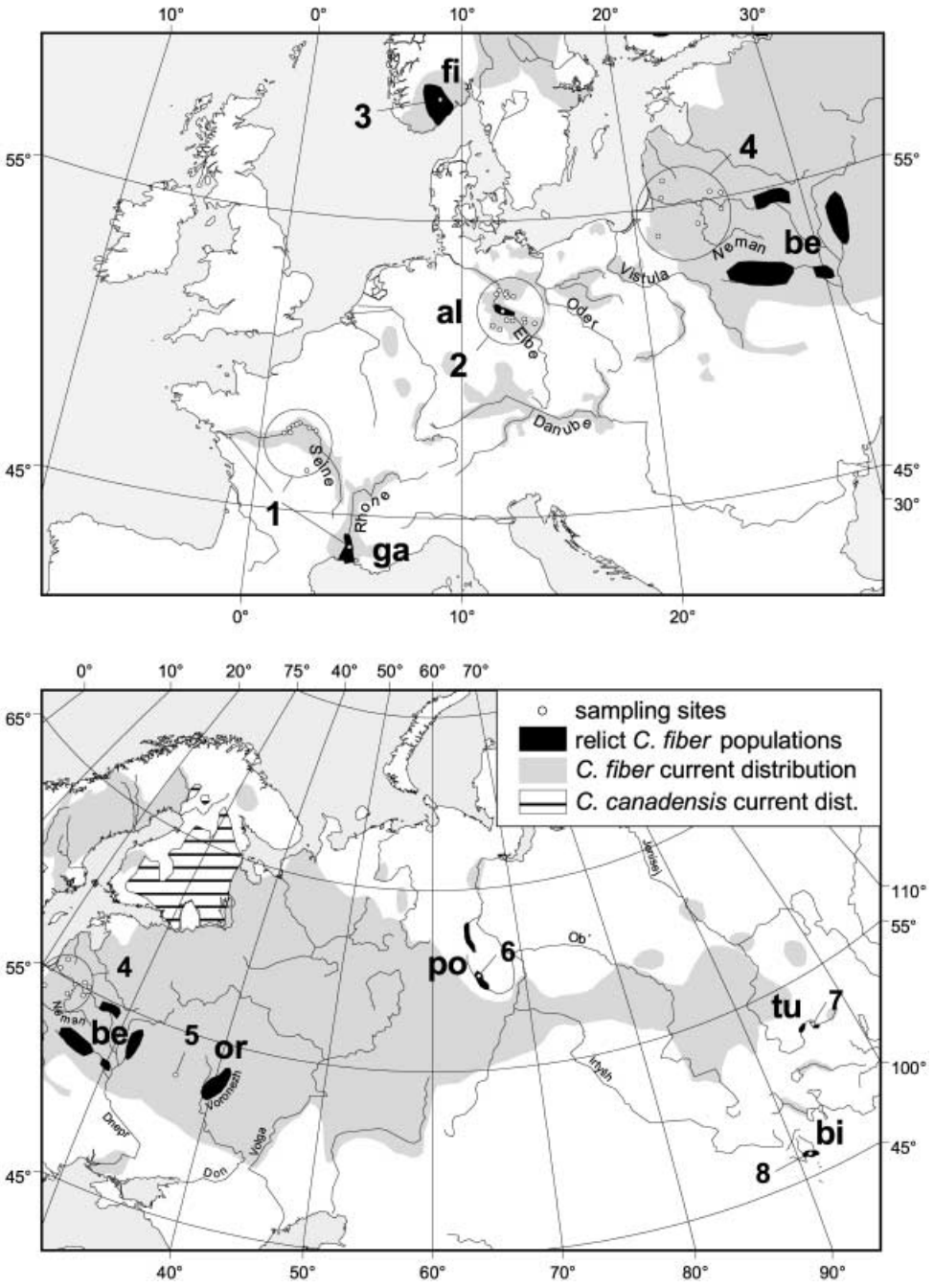


Fig. 1 Location of the sampling sites for *Castor fiber*. Current range of *C. fiber* and *Castor canadensis* according to Halley & Rosell (2002) and Saveljev (2003), except Russian far east. Relict populations remaining at the end of the 19th century: *C. f. albicus* (al), *C. f. belorussicus* (be), *C. f. birulai* (bi), *C. f. fiber* (fi), *C. f. galliae* (ga), *C. f. orientoeuropaeus* (or), *C. f. pohlei* (po) and *C. f. tuvinicus* (tu).

and the major histocompatibility complex (MHC), whereas Milishnikov *et al*. (1994, 1997) reported substantial genetic variation in beaver populations from the Russian Federation and Belarus using allozyme markers.

Currently, beaver management and conservation face three problems: (i) Although many beaver populations are expanding both geographically and demographically, Asian relict populations (*C. f. tuvinicus*, *C. f. pohlei*, *C. f. birulai*) remain small and static. Should these populations be managed independently? (ii) Eastern European beavers have the potential to spread into the previously isolated relict populations. The question arises whether such spread should be prevented. (iii) Reintroduction of beavers has been frequent without always taking into account historical genetic

Table 1 Samples of *Castor fiber* assessed for the sequence variation in the mtDNA control region fragment. Individuals are arranged according to their subspecific status (Lavrov 1981; Heidecke 1986) and geographical origin into eight populations (Fig. 1). *N*, number of individuals studied. Populations labelled '*C. f.* ssp. 1' and '*C. f.* ssp. 2' were sampled in the region where *C. f. belorussicus* and *C. f. orientoeuropaeus* hybridized, see text

No.	Subspecies/ population	Country	*N*	Haplotypes	Frequency	Localities
1	*C. f. galliae*	France	17	*ga1*	1.00	Bracieux, Candé, Chouzy, Menars, Onzain, Ouzouer, Vaucluse, Vertou Escrignelles, Lestioux, St-Laurent
2	*C. f. albicus*	Germany	27	*al1*	0.96	Bitterfeld (2), Dessau (4), Dübener Heide (1), Eisleben (1), Havelberg (2), Havelland (2), Mark Brandenburg (2), Merseburg (1), Schwarze Elster (8), Tangermünde (3)
				al2	0.04	Schwarze Elster
3	*C. f. fiber*	Norway	19	*fi1*	1.00	Bø i Telemark
4	*C. f.* ssp. 1	Poland/ Lithuania	21	*in2*	0.90	Poland: Pasłęka River, Kętrzyn (8); Lithuania: Giedraiciai, Kaunas, Kirsna, Raseinai, Šilute, Ukmerge, Molėtai, Žemaitija National Park
				in3	0.10	Kirsna, Kaunas (2)
5	*C. f.* ssp. 2	Russia	7	*in1*	1.00	Orël
6	*C. f. pohlei*	Russia	10	*po1*	0.90	Kondiskyj Zakaznik
				po2	0.10	Kondiskyj Zakaznik
7	*C. f. tuvinicus*	Russia	39	*tu1*	0.79	Azas River, Tyva Republic
				tu2	0.08	Azas River, Tyva Republic
				tu3	0.02	Azas River, Tyva Republic
				tu4	0.11	Azas River, Tyva Republic
8	*C. f. birulai*	Mongolia	12	*bi1*	0.17	Bulgan-Gol
				bi2	0.50	Bulgan-Gol
				bi3	0.33	Bulgan-Gol

population structure (but see Kitchener & Lynch 2000). How should future reintroductions be undertaken? These issues require information on the genetic structure of the species and, if possible, the definition of conservation units.

The concept of evolutionary significant units (ESU) for conservation has been proposed with focus either on presumably adaptive phenotypic divergence (Ryder 1986; Crandall *et al*. 2000; Van Tienderen *et al*. 2002) or on historically developed genealogical structure (Moritz 1994, 1999) of populations. It has been recognized that both concepts are complementary and that it is sometimes difficult to define categories within the continua of ecological and evolutionary variability of a species (Moritz 2002).

In an earlier study (Ducroz *et al*. 2005) we assessed the genetic variation and phylogeographical pattern in relict populations from the eastern part of the *C. fiber* range using mitochondrial DNA (mtDNA) control region sequences. An overall low level of genetic variation was coupled with an extremely high degree of genetic structure, concordant with the subspecific assignment of populations. Here we want to (i) explore the pattern of mtDNA variation in the aboriginal *C. fiber* populations over its entire range, (ii) gain insight into the population history of the beaver during the Pleistocene glaciations and routes of postglacial recolonization in Eurasia and put these finding in a broader context by comparison with other mammal species with similar distributions, and (iii) establish guidelines for future beaver translocations.

Materials and methods

Specimens examined

We analysed a total of 152 specimens of *Castor fiber* from 39 localities in France, Germany, Norway, Poland, Lithuania, Russia and Mongolia (Table 1 and Fig. 1). Sequences from 81 specimens representing the eastern part of the species' range were reported in an earlier study (Ducroz *et al*. 2005). Beavers originated either directly from sites where relict populations survived or from areas that are known to have been colonized solely from these. However, sampling the relict populations was not possible for the two eastern European subspecies *C. f. belorussicus* that had survived in the Dnepr watersheds in Belarus and the Ukraine and *C. f. orientoeuropaeus* that had survived in the Don and Voronezh watersheds in Russia. These populations were used for translocations and extensively hybridized (Milishnikov & Saveljev 2001). Additionally they could only be sampled in some distance from the relict sites (Fig. 1). Thus, we decided to consider the specimens sampled in these regions

as of indeterminate subspecific affiliation, and they will subsequently be labelled as '*C. fiber* ssp.'. Specimens from Poland and Lithuania were treated as representing one population due to their geographical proximity. All specimens were thus grouped into eight populations according to their geographical origin (Table 1). As an outgroup we used three sequences from *Castor canadensis* (GenBank Accession nos AY623644–6).

Laboratory analyses

Total genomic DNA was extracted following a modification of the cetyltrimethyl ammonium bromide (CTAB) method (Winnepenninckx *et al*. 1993) from small tissue fragments or hair preserved in 70% ethanol. The hypervariable domain I (HV I) (Saccone *et al*. 1987) of the control region (CR) of the mitochondrial genome was amplified using the universal primers Thr-L15926 (5′-CAATTCCCCGGTCTTGTAAACC-3′) and DL-H16340 (5′-CCTGAAGTAGGAACCAGATG-3′) (Cheney 1995; Vila *et al*. 1999). Additionally, for divergence time estimation we amplified the whole cytochrome *b* gene from six individuals using primers L7 (5′-ACCAATACC-AATGACATGAAAAATCATCGTT-3′) and H6 (5′-TCTCC-ATTTCTGGTTTACAAGAC-3′) (Montgelard *et al*. 2002a).

Polymerase chain reactions (PCR) were performed in 20- or 40-μL volumes. A 20-μL PCR mix contained 0.8 U of *Taq* polymerase (Fermentas), 2 μL of 10× *Taq* polymerase buffer with $(NH_4)_2SO_4$, 1.5 mM of $MgCl_2$, 200 μM of dNTPs and 5 pmol of each primer. PCRs for the CR used the following thermal cycling parameters: 4 min at 96 °C, 35 cycles (40 s at 96 °C, 45 s at 55 or 56 °C, 1 min at 72 °C) plus 10 min at 72 °C, whereas for the cytochrome *b* the cycling scheme was as follows: 4 min at 94 °C, 35 cycles (30 s at 94 °C, 30 s at 58 °C and 70 s at 72 °C) plus 7 min at 72 °C. PCR products were purified using the QIAquick PCR purification kit (QIAGEN) or the CleanUp kit (A&A Biotechnology) and eluted with 28 μL of water. Purified PCR products were directly sequenced in both directions using the same primers as for amplification. Approximately 20 ng of double-stranded PCR product was used in cycle sequencing reactions using the BigDye Terminator Cycle Sequencing Reaction Ready Kit 1.1 (Applied Biosystems). Dye terminators were removed using the DyeEx 2.0 Spin Kit (QIAGEN). Sequencing reaction products were separated on ABI PRISM 310 or 3100 automated sequencers (Applied Biosystems).

Phylogenetic analyses

The sequences were edited, managed and aligned with BIOEDIT 7 (Hall 1999) and CLUSTAL W 1.83 (Thompson *et al*. 1994). The alignments were optimized manually.

Phylogenetic relationships among the CR haplotypes were assessed using parsimony and distance methods. All analyses were performed with PAUP* 4.0b10 (Swofford 2003). In maximum-parsimony (MP) analysis, gaps were treated as a fifth character to account for the insertions/deletions (indels) present in this noncoding region. MP searches were conducted by the branch and bound method. A neighbour-joining (NJ) tree was constructed from the matrix of pairwise *p* distances. We deliberately used this simple distance measure, because it has low variance and thus, when sequence variation is low (as in our case), it is preferred for phylogeny reconstruction (Nei & Kumar 2000). Trees were rooted with three *C. canadensis* sequences. Robustness of both MP and NJ trees was tested with 1000 bootstrap replicates.

A median-joining network (MJ) (Bandelt *et al*. 1999) was used as another way of visualizing relationships among haplotypes. The MJ network was constructed with NETWORK4 (www.fluxus-engineering.com).

Population genetic analyses

Net sequence divergence (*Da*) between two major phylogroups and among populations, as well as nucleotide diversities (π) were computed with MEGA2 (Kumar *et al*. 2001); standard errors of the estimates were obtained through 1000 bootstrap replicates. Haplotype diversity (*h*) was computed with ARLEQUIN 2.001 (Schneider *et al*. 2000). Pairwise divergence for cytochrome *b* sequences and their standard errors (1000 bootstrap replicates) were computed with MEGA2.

We tested for the existence of population genetic structure using analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992) as implemented in ARLEQUIN. Pairwise differences between haplotypes were used as a molecular distance measure. The statistical significance of variance components in AMOVA was tested with 10 000 permutations.

The correlation between the geographical distance and population differentiation measured both as pairwise F_{ST} values and as the mean net number of pairwise differences was tested using the Mantel test as implemented in ARLEQUIN (10 000 permutations).

The extreme genetic structure with no haplotypes shared among populations (see below) did not allow for effective application of nested clade analysis, so the demographic history of the beaver phylogroups was assessed with two other approaches. Maximum likelihood (ML) based estimators of theta (θ_{ML}, $\theta = 2N_f\mu$ for mitochondrial genes, where N_f is the female effective population size and μ is mutation rate) and exponential population growth parameters (*g*) were computed jointly with FLUCTUATE 1.4 (Kuhner *et al*. 1998). This coalescent-based method takes into account genealogical relationships among haplotypes. The estimates of θ and *g* are obtained by Monte Carlo Markov chain searches through the genealogy space. The transition/transversion rate was set to 11.5 (estimated from the data), the rate of growth parameter change to 0.001, the Watterson (1975) estimator

Table 2 Condensed dot matrix displaying variables sites of the 495-bp alignment of the mtDNA control region for 16 haplotypes found in *Castor fiber*. Haplotype codes are given on the left, and nucleotide positions are displayed at the top; '–' denotes an indel

haplotype	nucleotide position 1111111122222222222233333333333344444444 3302234666114456789999011112346788112446 9 7123568201734014308345942479319651789769 90
bi1	GCAATAACTTTTCGAGTCCGCTGGTGAGTAATCGAATCGAAG
bi2	.....–...
bi3	–.....–...
po1	.....G.....A.A.T...C.....AC.G.....CT–...
po2	...C..G.....A.A.T...C.....AC.G.....CT–...
tu1	.....TA.A.TT....A.....G.T...CT–G.C
tu2	.....C.TA.A.TT....A.....G.T...CT–G.C
tu3	.....TA.A.TT....A....C.G.T..GCT–G.C
tu4	.....TA.A.TT....A.....GGT...CT–G.C
in1	–.....TG...TA.A.T.....G.....CT–.G.
in2	–.....TG...TA.A.....G.....CT–.G.
in3	–.....TG...TA.A.T.....A..G.....CT–.G.
al1	–AC....T.CCC.AGAC..AT...CA..C...TAGG.T–.G.
al2	–AC....T.CCC.AGAC..AT.A.CA..C...TAGG.T–.G.
fi1	–AC.CG.T.C..TA.A..TA.C..C..ACG..TA.G.T–.G.
ga1	–AC.....CC..A.AC..AT...CAGAC...TAGG.T–.G.

was used as a starting value of θ, and an UPGMA tree constructed from the matrix of *p* distances as a starting genealogy. We ran FLUCTUATE several times with different numbers of short and long chains to ensure consistency of the estimates. The final estimates were based on a run of five short chains of 10 000 steps each and one long chain of 100 000 steps; trees were sampled every 20 steps. The estimates of *g* are biased upwards (Kuhner *et al*. 1998) therefore following Lessa *et al*. (2003) a conservative approach in testing for significance was adopted, with values larger than three standard deviations (SD) of *g* regarded as significant.

The second approach was mismatch distribution analysis, following Schneider & Excoffier (1999). Mismatch analysis was shown to perform well in cases of population subdivision and when the demographic history of the populations involved is more complex than a simple model of sudden expansion (Rogers 1995). Computations were performed with ARLEQUIN. Goodness of fit to the sudden expansion model was tested using parametric bootstrap approach (10 000 replicates).

Results

Sequence divergence

Among 152 individuals of *Castor fiber*, 16 unique haplotypes were identified in the CR fragment. Twelve of them were reported in Ducroz *et al*. (2005) (GenBank Accession nos AY623632–43). The sequences of four new haplotypes were also deposited in GenBank (Accession nos DQ088700–03). The length of the fragment varied from 490 to 492 bp, with length variation attributable to single nucleotide indels. No large block insertions or variable number of repeats typical for this domain of the CR were present in our data set. Heteroplasmy was not detected, either in the length or in the nucleotide sequence of the fragment. Forty-two positions were variable and 34 parsimony informative. Excluding gaps 40 positions were variable and 33 parsimony informative (Table 2). All polymorphic sites exhibited two states. Nearly all substitutions were transitions (estimated ti/tv ratio: 11.5). The majority of variable sites were located at the 3'- end of the fragment (Table 2; sites 213–490).

The three sequences of *Castor canadensis* differed by one to six mutations, and were markedly shorter (473–474 bp) than in *C. fiber*. Including these *C. canadensis* haplotypes (*ca1*, *ca2*, *ca3*), the total length of the alignment was 495 bp, and the number of variable sites was 147, 138 of them being parsimony informative; excluding gaps 118 positions were variable, 111 parsimony informative.

Each haplotype was restricted to a unique geographical area, and consequently, no haplotypes were shared among subspecies (Table 1). All populations were characterized by one dominant haplotype with a frequency of at least 0.5. The highest number of haplotypes (four in *C. f. tuvinicus*) was found in a population with the largest number of individuals sampled, but the correlation between sample size and number of haplotypes was not significant ($r = 0.061$, $P = 0.88$). Haplotype diversity ranged from zero in *C. f. galliae* and *C. f. fiber* to 0.67 ± (SE) 0.09 in *C. f. birulai*. The overall level of genetic divergence within *C. fiber* was rather low, net sequence divergences among subspecies ranged from

	birulai	*pohlei*	*tuvinicus*	ssp.	*albicus*	*fiber*	*galliae*
birulai	0.0*	0.60	0.67	0.58	0.89	0.90	0.86
pohlei	2.06	0.04 (0.04)	0.60	0.54	0.93	0.82	0.86
tuvinicus	2.47	2.05	0.09 (0.05)	0.56	1.00	0.89	0.96
ssp.	1.87	1.71	1.74	0.12 (0.09)	0.87	0.81	0.86
albicus	4.32	4.73	5.09	4.14	0.02 (0.01)	0.73	0.44
fiber	4.32	3.91	4.30	3.69	2.88	0.0	0.70
galliae	4.12	4.12	4.89	4.31	1.03	2.67	0.0

*three haplotypes detected in *C. f. birulai* differed by indels only.

Table 3 Below diagonal: net sequence divergence (*Da*) between pairs of subspecies, based on *p* distance; above diagonal: standard errors of the estimates (1000 bootstrap replicates). On the diagonal: nucleotide diversities (π) within subspecies, with standard errors (1000 bootstrap replicates) in parentheses. All values are expressed as percentages

1.03% between *C. f. albicus* and *C. f. galliae* to 5.09% between *C. f. albicus* and *C. f. tuvinicus* (Table 3). Mean net divergence between *C. fiber* and *C. canadensis* was 18.27 ± 1.62% (22.85 ± 2.45% corrected for multiple substitutions according to the Kimura 2-parameter model).

Overall nucleotide diversity (π) within *C. fiber* was 2.9 ± 0.5%. Within subspecies π ranged from zero in *C. f. fiber* and *C. f. gallicus* to 0.09% in *C. f. tuvinicus* and 0.12% in *C. f.* ssp. (Table 3).

Six cytochrome *b* sequences represented five unique haplotypes (GenBank Accession nos DQ088704–08). Fourteen of 1140 nucleotide positions and five of 379 amino acid positions were variable. Pairwise divergence (*p* distance) ranged from zero between *C. f. albicus* and *C. f. galliae* to 0.7 ± (SE) 0.2% in comparisons: *C. f. birulai* vs. *C. f.* ssp., *C. f. birulai* vs. *C. f. fiber* and *C. f. pohlei* vs. *C. f.* ssp.

Phylogenetic relationships

Maximum-parsimony analysis of the CR sequences gave 16 most parsimonious trees (182 steps, CI = 0.863, RI = 0.931), their strict consensus together with bootstrap support (BS) values for particular nodes is given in Fig. 2a. Haplotypes from the three western subspecies: *C. f. albicus*, *C. f. fiber* and *C. f. galliae* formed a monophyletic group (85% BS), with *C. f. albicus* and *C. f. galliae* being sister groups with 95% BS. All other subspecies except for *C. f.* ssp. formed monophyletic units but relationships among them were not resolved.

In the NJ analysis (Fig. 2b) the western group was also recovered (95% BS). All eastern haplotypes tended to cluster together with moderate 81% BS. All eastern subspecies (including *C. f.* ssp.) emerged in the analysis as monophyletic units with BS from 85% to 100%. However, relationships among them remained unresolved. Average net sequence divergence between the eastern and western groups was 3.05% ± 0.63%.

The median-joining network shows clearly that the haplotypes from three western subspecies are close to each other, whereas the haplotypes from the eastern populations form four clusters connected by relatively long branches (Fig. 3).

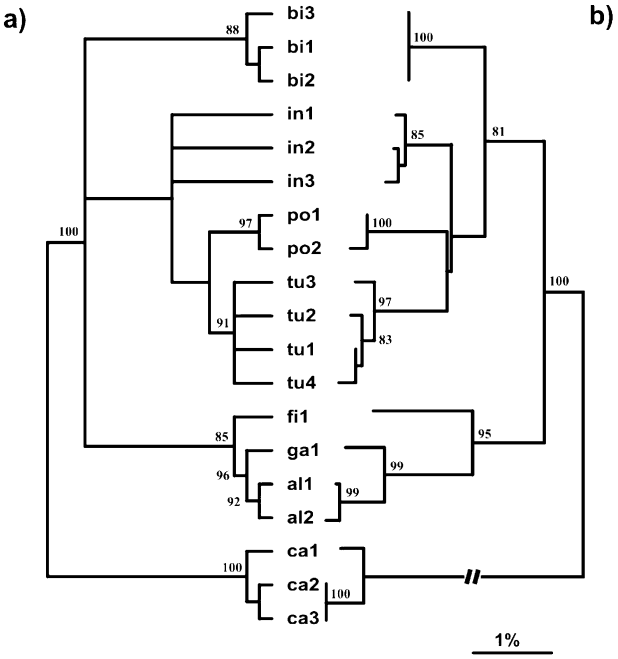


Fig. 2 (a) Strict consensus of 16 most parsimonious trees constructed from 16 *Castor fiber* mtDNA D-loop haplotypes; (b) neighbour-joining tree constructed from the matrix of pairwise *p* distances. Bootstrap values above 70% are shown; both trees were rooted with three *Castor canadensis* haplotypes.

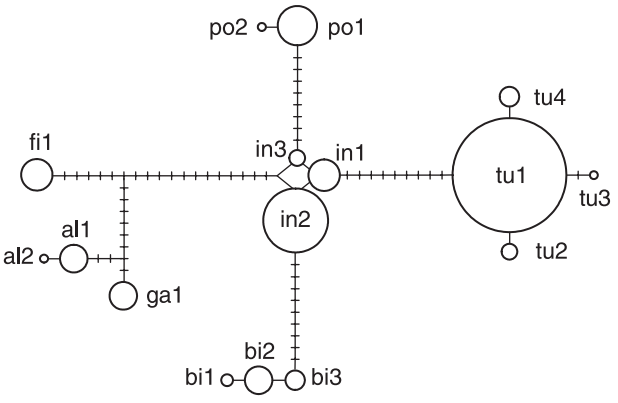


Fig. 3 Median-joining network for *Castor fiber* mtDNA control region haplotypes (Table 1). Circle areas are proportional to haplotype frequencies.

Population structure

The AMOVA revealed an extremely high level of genetic structure, with no CR haplotypes shared among populations. Almost all genetic variation (98.5%, $P < 10^{-5}$) was distributed among populations. Using a hierarchical model of population structure with populations grouped into eastern and western groups, 54.3% of variation was partitioned between groups and 44.6% among populations within groups. We did not find a significant correlation between the pairwise F_{ST} and geographical distances either among all populations or in the eastern phylogroup only (Mantel test, $P = 0.23$ and 0.24, respectively). Average net pairwise differences were marginally correlated with geographical distance when all the populations were included ($P = 0.04$).

Demographic analyses

Theta values from the FLUCTUATE analysis were larger in the eastern phylogroup [$\theta_{ML_east} = 0.0071 \pm$ (SD) 0.0010] than in the western group ($\theta_{ML_west} = 0.0036 \pm 0.0004$). In both groups the exponential growth parameter was negative ($g_{east} = -106 \pm 55$ and $g_{west} = -168 \pm 61$), although, under a conservative approach these were not significantly different from zero.

The distribution of the pairwise number of nucleotide differences did not conform to the model of sudden expansion in either of the groups (both $P < 10^{-6}$). In the eastern group, the distribution was bimodal with one, lower peak at zero, and the higher peak at nine pairwise differences (Fig. 4a). In the western group, the distribution was trimodal, reflecting the relatively high sequence divergence of *C. f. fiber* (Fig. 4b).

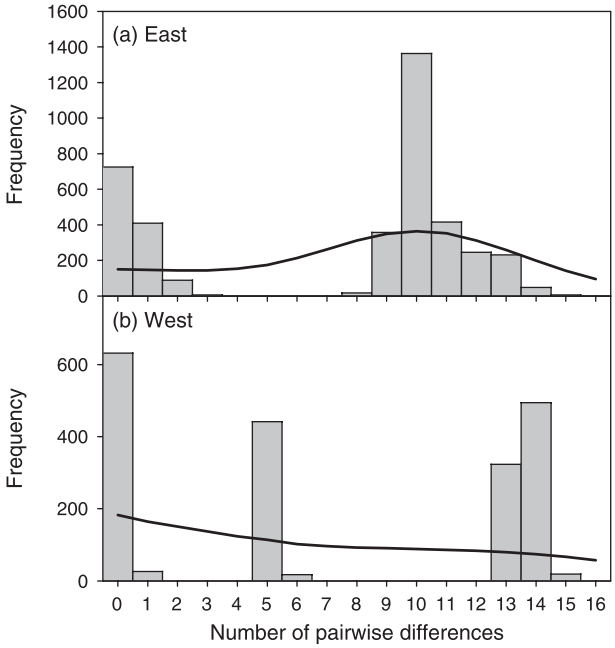


Fig. 4 Mismatch distributions for the eastern (a) and western (b) *Castor fiber* phylogroups. The black curve shows the expected distribution according to the sudden expansion model.

Discussion

Genetic variation within and between populations

Sequence variation of the assayed fragment of the mtDNA CR within the relict *Castor fiber* populations was very low. Particularly within the populations representing the western part of the range we found almost no variation (haplotype diversities in Norway and France, 0; in Germany, 0.07). This very low level of intrapopulation variation may be attributed to a recent bottleneck. Estimated population sizes at the beginning of the 20th century did not exceed 300 individuals in any of the relict populations and in France and the Russian Tuva Republic they could have been as low as 30 individuals (Lavrov 1969; Nolet & Rosell 1998; Halley & Rosell 2003). As the effective female population sizes were necessarily even lower, the strong action of genetic drift would have eliminated most of the mtDNA haplotypes. Low mtDNA variation has been repeatedly reported in taxa that has undergone severe bottlenecks (e.g. Houlden *et al*. 1999; Rosel & Rojas-Bracho 1999; Hellborg *et al*. 2002; Sinclair *et al*. 2002; Pang *et al*. 2003; Russello *et al*. 2004; but see Goldsworthy *et al*. 2000; Beheregaray *et al*. 2003; Godoy *et al*. 2004). In several cases, the impact of a bottleneck on the levels of variation was empirically validated with ancient DNA from prebottlenecked populations (Glenn *et al*. 1999; Weber *et al*. 2000; Larson *et al*. 2002; Nabata *et al*. 2004).

Our findings concerning the intrapopulational mtDNA variation should not be directly extrapolated to the nuclear genome. Whereas the Scandinavian beavers exhibit extremely low variation at multilocus minisatellite fingerprints and complete monomorphism at the MHC class I and II loci, the beavers from the European part of Russia show substantial polymorphism at minisatellite but not at MHC loci (Ellegren *et al*. 1993). In addition, allozyme variation is substantial in the Russian populations, both relict and reintroduced (Milishnikov *et al*. 1994, 1997; Milishnikov & Saveljev 2001; Milishnikov 2004). Further studies employing both neutral markers such as microsatellites, and genes of likely adaptive significance, as the MHC loci, are needed to assess the extent of the genetic variation in the beaver nuclear genome.

One of the most remarkable results of our study is the extreme genetic structuring within *C. fiber*. No CR haplotypes were shared between any pair of populations. This, together with close similarity of the haplotypes within

populations, raises the question if the pattern observed is a direct consequence of apparent cessation of gene flow following extirpation of the beaver from most of its former range, or if this pattern has deeper historical roots. It may be argued that following the postglacial expansion of the beaver and establishment of its historical range, populations evolved according to the isolation-by-distance model. Then, elimination of geographically intermediate populations would produce the observed pattern of extreme genetic structuring (see O'Ryan *et al*. 1998). However, it is also possible, and seems more likely, that the observed pattern is a result of a pre-existing genetic structuring of the beaver populations. Local populations of *C. fiber* have been shown to display pronounced genetic structure, even within watersheds (Milishnikov 2004). Furthermore, the beaver generally disperses along rivers (Halley & Rosell 2002) and thus the watersheds of great Eurasian rivers, Dnepr, Volga, Ob and Yenisej, could in the past have defined beaver populations, limiting gene flow among them. Moreover, in case of *C. f. fiber* the opening of the Skagerrak-Kattegat between northern Denmark and Norway *c*. 7500 BP (Björck 1995; Coyer *et al*. 2003) could have created effective barriers to gene flow. Finally, possible multiple glacial refugia in the eastern part of the range could also add to the complexity of the pattern (see below). Unfortunately, in the present case the clear-cut discrimination between the above-mentioned alternatives is difficult, although in general, comparative analysis of mtDNA variation across a species range may help to distinguish the effects of recent bottlenecks from these of prebottleneck genetic structure (Berry & Gleeson 2005).

Phylogeographical pattern and Pleistocene history

Castor fiber shows a clear phylogeographical pattern across its historical range with the western populations from Germany, France and Norway clustering together and distinct from populations east to the Oder and Vistula rivers.

The application of a molecular clock for the mtDNA CR is controversial, because of high variation in the rate of sequence evolution within and between domains of the CR and because of high rate heterogeneity observed among mammalian taxa (Sbisa *et al*. 1997; Excoffier & Yang 1999; Pesole *et al*. 1999; Heyer *et al*. 2001; Larizza *et al*. 2002). Therefore we used cytochrome *b* sequence divergence to set an approximate time frame on the age of mtDNA lineages. Even among rodent lineages there is a substantial heterogeneity in the cytochrome *b* substitution rates (Spradling *et al*. 2001; Montgelard *et al*. 2002b). Thus we apply two calibrations: slow, 3.04% divergence per million years (Myr) (Eddingsaas *et al*. 2004) and fast, *c*. 10–12% (She *et al*. 1990; Arbogast *et al*. 2001). The slow calibration gives the age of the oldest mtDNA lineages of 0.23 Myr (95% confidence intervals: 0.10–0.36 Myr) and the fast calibration of 0.06 Myr (CI: 0.02–0.09 Myr). It is the rule that for recent divergences the age of allelic lineages exceeds that of populations due to retention of ancestral polymorphism (Edwards & Beerli 2000; Arbogast *et al*. 2002). Accordingly we postulate the separation of beaver populations during the last glacial period that started 115 000–110 000 BP (Tzedakis 2003).

The existence of two major phylogroups indicates at least two glacial refugia. However, their location is extremely difficult to reveal. Subfossil data and historical records indicate that the Holocene range of the beaver included areas of the widely recognized Pleistocene refugia: the Iberian, Italian and Balkan peninsulas (Boessneck 1974; Nolet & Rosell 1998). The west Mediterranean region constitutes the potential refugium for the western phylogroup. Examples of successful postglacial colonization of major parts of Europe from each of these refugia have been described (Taberlet *et al*. 1998; Hewitt 1999, 2004). Refugia of the eastern phylogroup could be located in the Balkan Peninsula, the Pontic region, in the Near East or further to the east (Vereshchagin & Burchak-Abramovich 1958; Boessneck 1974). It is also possible, given that we found no support for the eastern group monophyly in the parsimony analysis and relatively long branches connecting its haplotypes in the MJ network (Figs 2 and 3), that in the eastern part of its range the beaver survived the last glaciation in several refugia, or in a more or less continuous belt of suitable environments ranging from eastern Europe to central Asia. A growing body of evidence supports the presence of forest vegetation on the shores of the Azov and Black seas, in southern Urals, in southern Siberia and Mongolia during the last glacial maximum (Efimik 1996; Tarasov *et al*. 2000; Payette *et al*. 2002; Kuzmin & Orlova 2004). Phylogeographical analyses of several mammalian taxa point to the importance of these areas as glacial refugia (Taberlet & Bouvet 1994; Bilton *et al*. 1998; Jaarola & Searle 2002; Brunhoff *et al*. 2003). More precise information on the location of the beaver glacial refugia could be gained from the genetic analysis of subfossil or museum material from the above-mentioned regions.

The eastern European beaver survived the 19th century bottleneck in the Don and Dnepr watersheds and subsequently recolonized large areas both spontaneously and by human translocations followed by hybridization. The mtDNA haplotypes of '*C. f.* ssp.', originating from sites recolonized by *C. f. belorussicus* and *C. f. osteuropaeus*, closely clustered together (Fig. 3). Thus, our data imply that these two relict populations were not strongly differentiated genetically and originated from the same glacial refugium.

The east–west split between mtDNA lineages observed in the beaver in central Europe is paralleled by similar splits in a number of other species, including mammals (Boursot *et al*. 1993; Taberlet & Bouvet 1994; Jaarola & Searle 2002; Brunhoff *et al*. 2003; Deffontaine *et al*. 2005), amphibians

(Babik *et al*. 2005), freshwater fishes (Durand *et al*. 1999; Nesbo *et al*. 1999) and insects (Stauffer *et al*. 1999). The exact location of contact zones varies, ranging from Germany to Lithuania, probably reflecting a number of factors, such as geographical location of glacial refugia, availability of migration corridors and relative speed of colonization of the European plains (Ibrahim *et al*. 1996; Hewitt 1999). Further to the east, the Urals seem to be also an important phylogeographical boundary, separating genealogical lineages in several species (e.g. Jaarola & Searle 2002; Brunhoff *et al*. 2003; Deffontaine *et al*. 2005). Within the eastern phylogroup of the beaver, the Urals geographically separates the eastern European beavers from the central Asian subspecies and may have contributed to the pronounced genetic structure.

Demographic history

Dramatic perturbations in historical times must have affected profoundly both population structure and levels of genetic variation, rendering analyses of demographic history of the beaver phylogroups difficult. Of course, both historical events and recent effects related to beaver extirpation will be reflected and virtually impossible to separate in these analyses. Therefore the results should be treated with caution.

The effective female population size estimated from the CR sequence variation seems to be larger in the eastern phylogroup, likely as a result of a higher number of relict populations that preserved a higher fraction of the mtDNA genetic variation formerly present in this group. It remains an open question if prebottleneck effective female population size was also higher in this group. The genealogy-based FLUCTUATE analysis and mismatch distribution suggest demographic contraction in both phylogroups, in line with historical evidence. The multimodal shape of the mismatch distributions in both groups reflects genetic distinctness of individual populations and subspecies.

Beaver conservation genetics

Current population expansion of the Eurasian beaver and future reintroduction plans raise the concern about how populations should be managed. In our reasoning about conservation units, we focus on the concept and criteria on neutral mtDNA marker differentiation put forward by Moritz (1994, 1999, 2002). Such an approach should be complemented by investigations on ecological and adaptive distinctness (Crandall *et al*. 2000), but this is beyond the scope of our study (see, e.g. Rosell & Steifetten 2004). All relict beaver populations (except *C. f.* ssp.) have been demographically independent for centuries and currently do not share mtDNA haplotypes, and thus qualify as management units (Moritz 1994). The mtDNA haplotypes form a hierarchical set of reciprocally monophyletic units (Fig. 2). First, the eastern and the western populations are characterized by reciprocally monophyletic mtDNA clades in the NJ analysis, and thus qualify as ESUs *sensu* Moritz (1994) and deserve highest conservation priority. Second, all populations emerge as reciprocally monophyletic in the neighbour-joining tree. However, the criterion of reciprocal monophyly may be oversensitive (Moritz 1994; Paetkau 1999; Crandall *et al*. 2000). Reciprocal monophyly in mtDNA could arise relatively quickly after population subdivision and may take ~$4N_e$ generations, where N_e is the individual effective population size of two equally sized units after fragmentation (Neigel & Avise 1986). In beaver, the minimum population size was between 30 and 300 in all relict populations (Nolet & Rosell 1998). Thus it may be argued that reciprocal monophyly of the populations within the eastern and western clade could have developed in the last centuries as a result of human population subdivision and drastic population bottlenecks rather than long-lasting independent evolution (Ducroz *et al*. 2005). However, given the large distances among the eastern subspecies relative to the western ones it might be considered whether the eastern subspecies deserve the status of ESUs. In conclusion, the mtDNA data presented here suggest that there are at least two ESUs: the western phylogroup (*C. f. gallicus*, *C. f. albicus* and *C. f. fiber*) and the eastern phylogroup (*C.f.* ssp., *C. f. tuvinicus*, *C. f. pohlei*, *C. f. birulai*). As long as further information on other types of molecular markers or phenotypic traits is lacking, the individual relict populations should be treated as management units (MUs), which may be referred to by their subspecific taxonomic ranks.

Concerning reintroductions, clear recommendations can be formulated from our results: for reintroductions in western and central Europe, only western beavers (*C. f. gallicus*, *C. f. albicus* and *C. f. fiber*) of known origin should be used, whereas only eastern beavers should be used for reintroductions east of the Oder and Vistula rivers. Within both regions, the geographically nearest form should be used for reintroductions unless the genetic identity of the original population is known (IUCN/SSC 1995). Additionally, in order to keep the identity of populations, animals should originate from one subspecies only. Because of their current small population size (Stubbe *et al*. 1991), *C. f. tuvinicus*, *C. f. pohlei*, and *C. f. birulai* deserve special conservation efforts in order to stabilize their populations which may come into contact with the expanding eastern European beavers.

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