

# First molecular evidence on the puzzling origin of beavers in central Italy

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## Short title

Tracing beaver origin in Italy through mitochondrial DNA

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## ABSTRACT

The Eurasian beaver (*Castor fiber*) represents an extraordinary example of demographic recovery after centuries of severe hunting and extirpation. Reintroduction programs and legal protection have triggered the recolonisation of Europe, with rapid expansion of beaver populations in the last 25 years. In northern Italy, the species reappeared in 2018 after a 450-years absence, due to natural spreading of individuals from neighbouring countries. Since 2021, the presence of beavers has been reported in several areas of central Italy, probably following unofficial releases. Here, we employed mitochondrial DNA markers, mainly from non-invasive samples, to rule out the presence of the North American beaver (*C. canadensis*) and to characterise both the beavers from northern Italy and the new population from central Italy, assessing their genetic relationships with other European beavers. The analysis of 35 samples from Italy and other countries provided no evidence of the North American sister taxon and revealed four widely distributed mitochondrial haplotypes, which are discussed in the current framework of genetically mixed populations in Europe. In addition, this note reports genetic data from beavers in the Balkans, where no information has been available so far.

The Eurasian beaver (*Castor fiber* L., 1758) is a rodent that was once widespread and abundant throughout Eurasia (Halley et al., 2021). Since the Middle Ages, over-hunting and habitat loss caused severe demographic reductions and extinctions from vast areas of its former range, including Italy (Salari et al., 2020). Early in the 1900s, the species survived only in scattered relict populations, in Asia, but mainly in Europe (Halley et al., 2021). After this severe bottleneck, over the last decades, beavers recolonised most of their historical range following reintroduction programs and legal protection (Halley et al., 2021). Translocations of individuals, both legal and unofficial, have been essential for the survival of the species in Europe. However, these were conducted with animals from various geographical origins (Frosch et al., 2014; Senn et al., 2014; Durka et al., 2005), leading to a complex pattern of genetically mixed populations, and somewhere (e.g., in Finland; Iso-Touru et al., 2021) causing the establishment of the North American beaver *C. canadensis* Kuhl, 1820 (Halley et al., 2021; Minnig et al., 2016; Biedrzycka et al., 2014; Parker et al., 2012; Dewa et al., 2012). In several cases, molecular methods have been employed both to disentangle the origin of the newly observed beaver populations and to identify *C. canadensis* when field observations were inconclusive (Munclinger et al., 2022; Frosch et al., 2014; Kropf et al., 2013; Kuehn et al., 2000).

In Italy, the species reappeared in 2018 after a 450-years absence, in the northeast of the country, because of natural spreading from the reintroduced populations in Austria and Switzerland (Halley et al., 2021; Pontarini et al., 2019). The presence of at least four individuals, which crossed the Italian borders, has been documented since 2018 in Tarvisio, province of Udine, and Val Pusteria, Bolzano (Righetti et al., 2023; Pontarini et al., 2019). Furthermore, unequivocal beaver signs have been recorded in 2021 in central Italy (Pucci et al., 2021). The presence of beavers was later confirmed through camera-trapping of several individuals in two areas of Tuscany: the Ombrone-Merse river basin (provinces of Siena and Grosseto) and the upper Tiber valley (province of Arezzo), as well as in Umbria (still in the Tiber river), with evidence of at least three years of reproduction (Viviano et al., 2022; Mori et al., 2021; Pucci et al., 2021). Given the large geographic distance from the northern areas of beaver occurrences, natural spreading of individuals towards central Italy was unlikely. Although their origin is controversial, illegal reintroductions, however, are hypothesised as the most likely scenario (Pucci et al., 2021). In this study, we employed mitochondrial DNA (mtDNA) to examine beavers from northern Italy and the newly discovered population in central Italy. Non-invasive samples (faeces) and tissues from dead beavers collected in Italy, as well as neighbouring countries, were analysed genetically with two aims: 1) to rule out the possible presence of the North American beaver, as was the case of other European areas (Dewas et al., 2012; Kropf et al., 2013); 2) to characterise northern beavers and the new population in central Italy, by evaluating their genetic relationships with other European beavers.

Seventeen biological samples were collected in 2022 from Italy, supplemented by samples from Switzerland (n=2), Austria (n=8), and the Balkans (n=8) (Tab.1, Fig.1). Fourteen faecal samples and one tissue were from central Italy, two faecal samples were from the known areas of occurrence in northern Italy (Tarvisio, Val Pusteria), and 18 tissues were collected from other European countries. Faeces were stored dry at -20°C and tissues were stored in ethanol 96-100%. Total genomic DNA was isolated from faeces using the Maxwell16 Instrument (Promega, Madison, USA), while tissues were processed with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following producer's handbook.

Two pairs of new species-specific primers were developed for the amplification and sequencing of mitochondrial markers in beavers. Primers were designed using Primer3 (<https://primer3.ut.ee/>) and Primer-BLAST web tools (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). A first primer pair amplified a fragment of 120 base pairs (bp) in the Cytochrome b (Cytb\_Castor: F 5' -ACATCAGACACCACAACAGC - 3'; R 5' - TCCGTAGTAGATTCCTCGTCC - 3'), which was used to differentiate the American beaver from its Eurasian sister species. A second pair amplified a 330 bp fragment in a highly variable section of the Control Region (CR\_Castor: F 5' - ACAACAGTCTATGTATATCGTGCAT - 3'; R 5' - GGAGCGAGAAGAGGGACATT - 3'), which was used to detect mitochondrial lineages within the Eurasian beaver. PCR conditions were the same for both primer sets: an initial activation step at 94°C for 3 min; 38 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 30

s, followed by a final extension of 5 min at 72°C. Negative and positive controls were included to check success of the reactions and monitor contaminations. Sequencing was performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), then products were loaded on ABI 3130 Genetic Analyzer (Applied Biosystems). Cytb sequences were obtained only for faecal samples, to exclude the presence of the American beaver, while the CR fragment was obtained for both faeces and tissue samples. DNA sequences were checked manually and aligned with Geneious Prime 2022 (<https://www.geneious.com>). The software DnaSP 6.0 (Rozas et al., 2017) was used to determine the number of haplotypes for both Cytb and CR sequences. The CR haplotypes obtained were analysed along with 31 DNA sequences downloaded from GenBank, covering the current Eurasian range of *C. fiber* (Frosch et al., 2014; Senn et al., 2014; Kropf et al., 2013; Horn et al., 2011; Ducroz et al., 2005; Durka et al., 2005). Compared to previous studies (Durka et al., 2005; Senn et al., 2014), our alignment covered a shorter, albeit highly informative, CR fragment (330 bp vs. approximately 490 bp). A median-joining network (Bandelt et al., 1999) was constructed using the software PopArt (Leigh and Bryant 2015) to visualise the relationships among CR haplotypes.

The new primers yielded successful amplifications both in tissues and non-invasive samples, except for three samples from the Merse river (Tab.1). We obtained Cytb sequences from 13 faecal samples and CR sequences from 32 faecal and tissue samples. All Cytb sequences yielded the same haplotype matching a *C. fiber* sequence from Germany (Genbank accession number AF155879; Kuehn et al., 2000). Consequently, no evidence of *C. canadensis* was found in our beaver samples.

Of the 32 CR sequences obtained, we detected four different haplotypes that were aligned and compared with 31 homologous sequences available from GenBank. Following the nomenclature in Munclinger et al. (2022), we identified the haplotype JF7, previously found in the Voronezh relict population from Russia as reported by Senn et al. (2014), the haplotypes ga1 and fi1, first described by Durka et al. (2005) in France and Norway respectively, and the haplotype EDFiba03, first reported in Austria, but probably also originating from Norway (Kropf et al., 2013). In beavers from Austria, we found all four haplotypes, of which only three (JF7, fi1, EDFiba03) had been previously retrieved by Kropf et al. (2013) in this area. In Switzerland, we found ga1 and fi1, in accordance with Minnig et al. (2016). There are no data in the literature for the Balkan countries, hence, both the presence of JF7 in Serbian and Croatian populations, and ga1 at a single site in Bosnia and Herzegovina, are new reports. In northern Italy, where beavers probably spread from nearby areas (Righetti et al., 2023; Pontarini et al., 2019), we found ga1 in both sampled localities (Tarvisio and Val Pusteria), consistently with the presence of this haplotype in Austria. The newly-founded population in central Italy showed two haplotypes: JF7 dominated in different localities of Tuscany, while ga1 was found in one beaver sample from Ombrone river (Tuscany) and one sample from Promano (Tiber river, Umbria). These haplotypes are considered being native to Russia (Voronezh) and France, respectively (Senn et al., 2014; Durka et al., 2005), but currently they are widespread across Europe (Minnig et al., 2016; Biedrzycka et al., 2014; Frosch et al., 2014; Kropf et al., 2013; Horn et al., 2011; Munclinger et al., 2022).

Previous phylogeographic studies indicated that the CR haplotypes found in the extant Eurasian beaver populations were arranged into two divergent monophyletic haplogroups, referred to as western and eastern lineage, respectively (Durka et al., 2005). However, contrary to what was initially hypothesised, further studies suggested that the lineages were not as divergent as laid out, nor that there was clear correspondence to geographic populations (Senn et al., 2014; Munclinger et al., 2022). A mixture of haplotypes from both lineages are present in many of the current beaver populations (Senn et al., 2014). Multiple bottleneck events in populations retreating into refugia during the Last Glacial Maximum (approximately 25 000 ya), followed by secondary contacts of re-expanding populations, might be a sound explanation of beaver phylogenetic history in Eurasia (Senn et al., 2014). When our CR sequences were

added to published sequences to build a network, the two lineages were still clearly visible (Fig. 2), and all beavers analysed in the present survey were grouped into the western lineage.

This study is the first molecular investigation on beavers from Italy through non-invasive sampling and analysis of mtDNA. Our findings suggest that the newly established population (Pucci et al., 2021, Mori et al., 2021) was very likely founded by a genetically mixed stock, as described from other areas in Europe. In particular, individuals reintroduced in central Italy bore two haplotypes that are currently widely shared and distributed both in western and eastern populations of *C. fiber* (e.g., JF7) (Munclinger et al., 2022; Frosch et al., 2014). Moreover, we found no evidence for the presence of the North American beaver in Italy. Further genetic investigations through detailed assessment of variation at the nuclear level, will contribute to clarifying their origin, proving estimates of the minimum number of individuals in each area, and the reconstruction of kinship relationships in a management and conservation perspective.

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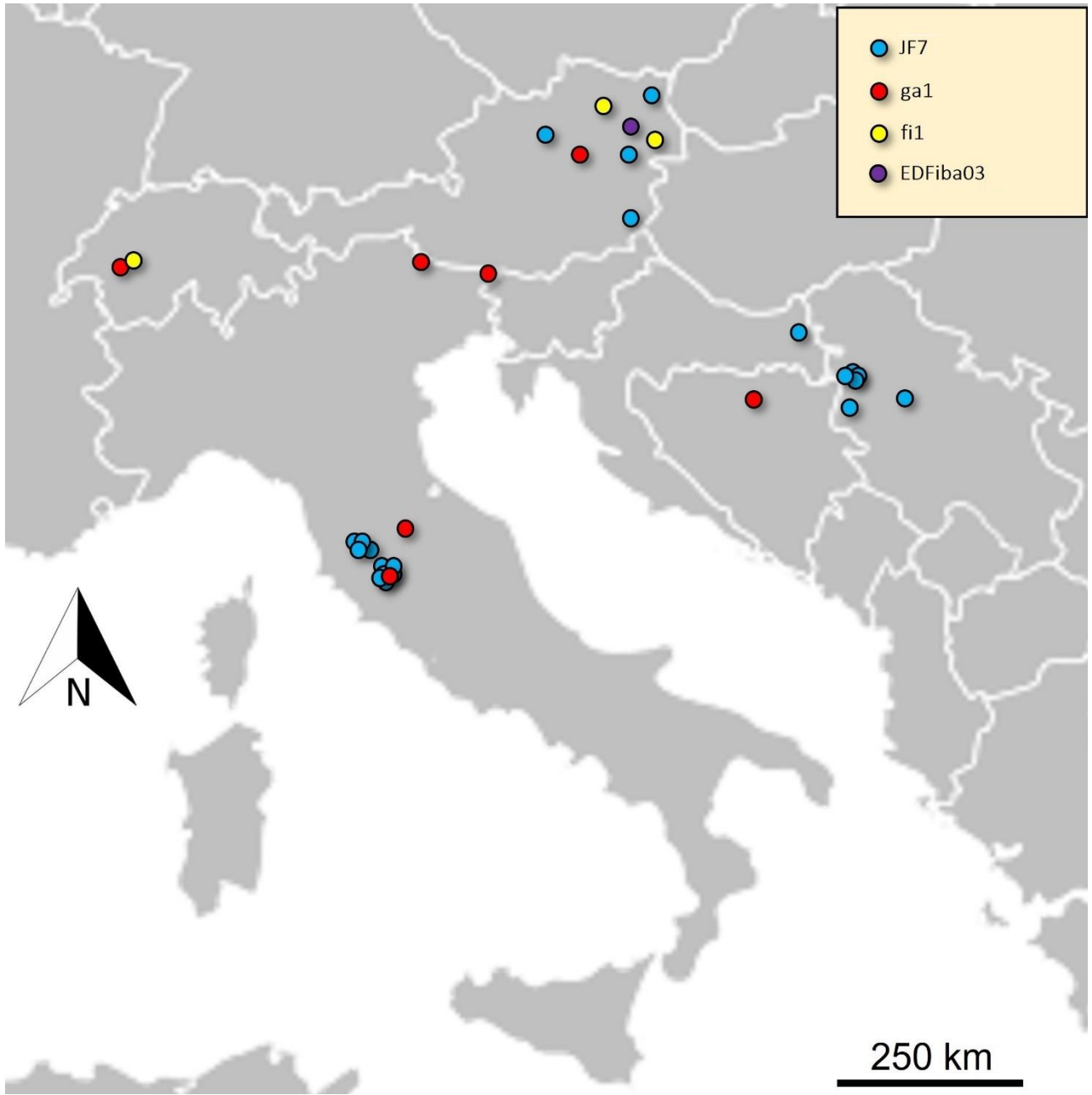
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**Tab. 1** Samples of *Castor fiber* analysed in this study. F = faeces, T = tissue. Haplotype nomenclature follows Munclinger et al. (2022). NA = not amplified.

Sample ID	Locality (Country)	Biological material	CR haplotype
Merse 1	Risaie, Merse river, Siena (Italy)	F	NA
Merse 2	Monticiano, Merse river, Siena (Italy)	F	NA
Merse 3	Monticiano, Merse river, Siena (Italy)	F	NA
F1	Casal di Pari, Ombrone river, Grosseto (Italy)	F	JF7
F4	Casal di Pari, Ombrone river, Grosseto (Italy)	F	JF7
F5	Casal di Pari, Ombrone river, Grosseto (Italy)	F	JF7
FD1	Casal di Pari, Ombrone river, Grosseto (Italy)	F	JF7
FD2	Casal di Pari, Ombrone river, Grosseto (Italy)	F	ga1
Ombrone 1	Casal di Pari, Ombrone river, Grosseto (Italy)	F	JF7
CAS1	Casal di Pari, Ombrone river, Grosseto (Italy)	F	JF7
CAS3	La Befia, Ombrone river, Siena (Italy)	F	JF7
CAS4	Il Doccio, Merse river, Siena (Italy)	F	JF7
CFIB1	Ponte Macereto, Merse river, Siena (Italy)	F	JF7
V1	Orgia, Merse river, Siena (Italy)	F	JF7
OT1	Promano, Tiber river, Perugia (Italy)	T	ga1
Tar1	Tarvisio (Italy)	F	ga1
VP1	Val Pusteria (Italy)	F	ga1
S1	Zasavica (Serbia)	T	JF7
S2	Doboj (Bosnia and Herzegovina)	T	ga1
S3	Donje Nedeljice (Serbia)	T	JF7
S4	Zasavica (Serbia)	T	JF7
S5	Kopačevo (Croatia)	T	JF7
S6	Zasavica (Serbia)	T	JF7
S7	Zasavica (Serbia)	T	JF7
S8	Obrenovac (Serbia)	T	JF7
SWISS_1	Canton of Fribourg (Switzerland)	T	ga1
SWISS_2	Canton of Fribourg (Switzerland)	T	fi1
AT-Cf-01	St. Pölten, Wasserburg (Austria)	T	EDFiba03
AT-Cf-02	Fürstenfeld, Bad Loipersdorf (Austria)	T	JF7
AT-Cf-03	Aist, Mühlbach (Austria)	T	JF7
AT-Cf-04	Leithaprodersdorf (Austria)	T	fi1
AT-Cf-05	Zistersdorf (Austria)	T	JF7
AT-Cf-06	Gaming (Austria)	T	ga1
AT-Cf-07	Hadersdorf (Austria)	T	fi1
AT-Cf-08	Muggendorf, Myrabach (Austria)	T	JF7

**Figure 1** Map of sampling sites. Localities are described in detail in Tab.1.



**Figure 2** Median-joining network of mitochondrial CR haplotypes in beavers. E = eastern lineage, W = western lineage according to Durka et al. (2005). The two haplotypes found in central Italy are marked in blue and red.

